Modulation of the sensitivity of nociceptive neurones by cannabinoids and SNSRs

A thesis submitted for the degree of Doctor of Philosophy

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The sensation of pain is evoked when the body experiences noxious stimuli, and such stimuli are detected by specialised primary sensory neurones called nociceptors. Modulation of the sensitivity of these nociceptors occurs physiologically when they are exposed to inflammatory exudates. A fuller understanding of the means by which these neurones are modulated will provide relevant information for the rational development of analgesics.

The receptors for cannabinoids, the active ingredients of cannabis, have been found in some primary sensory neurones. Given the known ability of these receptors to inhibit adenylate cyclase, the activation of which leads to the sensitisation of nociceptors, calcium imaging was used to assess if the application of a synthetic cannabinoid agonist (WIN 55,212-2) would attenuate the responsiveness of nociceptors *in vitro*. After examining this in both a non-sensitised model and a model of sensitisation employing prostaglandin E_2 (PGE_2), it was evident that, in the *in vitro* model employed, the method of activating cannabinoid receptors did not result in any significant effect.

Another family of G-protein coupled receptors found exclusively in sensory neurones (Sensory Neurone Specific Receptors or SNSRs) were then examined to see if the application of their agonist, bovine adrenal medulla peptide (BAM (8-22)), modulated the response of nociceptors *in vitro*. A sub-population of neurones was seen to sensitise in their response to capsaicin after exposure to BAM (8-22). A selection of kinase inhibitors was then employed to elucidate the intracellular pathway involved in this sensitisation, and protein kinase C (PKC) was found to play a significant role. The specific PKC-isoform involved, however, was not able to be elucidated using immunocytochemistry. Whole cell patch clamp studies both confirmed the findings from the calcium imaging study, and established that the transient receptor potential vanilloid receptor itself was sensitised by the application of BAM (8-22).

Declaration

All the experiments and results described in this dissertation, except where otherwise stated, are my own work, and were carried out in the Department of Pharmacology, University of Cambridge, between October 2000 and June 2004. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation is not substantially the same as any that I have submitted for a degree or diploma or other qualification at this, or any other University. This dissertation contains work that was submitted in 2001 for the Certificate of Postgraduate Study in Natural Science (Biological Science), apart from that no other part of it has already been, or is being currently, submitted for any such degree, diploma or other qualification. This dissertation does not exceed the prescribed limit of 300, single-sided, pages of double spaced text, not including the bibliography and appendices.

Stuart Andrew Honan

28th June 2004

Acknowledgements

To those beacons of light when the seas were rough

Where does one begin, especially with a large lab group and many changes? I would like to thank my supervisor Peter McNaughton, who has provided guidance and helpful comment on my thesis, as well as tolerating my perfectionist tendencies. The lab member to whom I owe most is my fellow PhD student Anna Kinsey. As students together we went through the rollercoaster ride that is the PhD, with highs and lows. If I had not had Anna as a source of advice in the difficult times the three years would have certainly led to insanity. The list of those who have passed through the lab and have made important contributions is long indeed. In the beginning, Jenny Bonnington, as she was finishing her studies, taught me about culturing DRG neurones and calcium imaging, and Colin Blackmore provided much support when I was learning to use the confocal microscope. I would like to thank Erica Sharpe, Herve Cadiou and Laura Bobanovic for their assistance with patch clamping. Vittorio Vellani for more patch clamping advice, and, along with Mariantonella Colucci, for help with the PKC translocation assay. And finally, the lab technicians who provided essential support to ensure that the wheels never quite came off: Helen Bye, Cathy Day and Andy Charlton.

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Abbreviations

5-HT	5-hydroxytryptamine
α,β-Me-ATP	α,β-methyleneadenosine 5'-triphosphate
AEA	(see anandamide)
AHP	afterhyperpolarization
anandamide	arachidonyl ethanolamide
ANOVA	analysis of variance
Ara-C	cytosine β-D-arabinofuranoside
ATP	adenosine triphosphate
BAC	bacteria artificial chromosome
BAM (8-22)	8-22 fragment of BAM 22
BAM 22	bovine adrenal medulla peptide 22
cAMP	cyclic 3',5'-adenosine monophosphate
CB_1	cannabinoid receptor 1
CB_2	cannabinoid receptor 2
cDNA	complementary deoxyribonucleic acid
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclo-oxygenase
Δ^9-THC	Δ^9-tetrahydrocannabinol
DAG	1,2-diacylglycerol
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DRG	dorsal root ganglion
DRGs	dorsal root ganglia
EC_{50}	half-maximal effective concentration
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EP3C	prostaglandin E_2 receptor 3C
EP4	prostaglandin E_2 receptor 4
FBS	fetal bovine serum
GDNF	glial cell line-derived neurotrophic factor
GPCRs	G-protein coupled receptors
GTP	guanosine triphosphate
H-89	N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl
HBSS	Hank's balanced salt solution
HEK293	human embryo kidney 293
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IB_4	isolectin B4
IP_3	inositol 1,4,5-trisphosphate
MRG	*Mas*-related gene
mRNA	Messenger ribonucleic acid
NGF	nerve growth factor
NO	nitric oxide
PEA	palmitoylethanolamide
PGE_2	prostaglandin E_2

PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
POMC	pro-opiomelanocortin
PTP	protein tyrosine phosphatase
RF-amide	arginine-phenylalanine-amide
RNA	Ribonucleic acid
Ro-31-8220	3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide
s.e.m.	standard error of the mean
SNSR	sensory neurone specific receptor
TNF α	tumour necrosis factor α
TRPV1	transient receptor potential vanilloid 1 cation channel
TTX-R	tetrodotoxin-resistant
WIN 55,212-2	(R)-(+)-[2,3-dihydro-5-methyl-3[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate

Chapter 1

Introduction

1.1 Pain: A general overview

Pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP Task Force on Taxonomy, 1994). It is an important sense that helps to prevent or limit damage to the body, and thus performs an essential role in survival. As a sensation experienced by all of us it has been of interest to humanity for centuries whether through means to alleviate it or through attempts to describe the mechanism by which noxious stimuli result in it. An attempt by René Descartes to describe this mechanism in 1644 went as follows: "If for example the fire…comes near the foot…, the minute particles of this fire, which as you know move with great velocity, have the power to set in motion the spot of the skin of the foot which they touch, and by this means pulling on the delicate thread…they open up at the same instant the pore…against which the delicate thread ends, just as by pulling against one end of a rope one makes to strike at the same instant a bell which hangs at the other end". This description relates the communication of a noxious stimulus via an internal physiological means to the brain, in which the striking of the bell can be correlated with the emotional sensation of pain. Nowadays, we are aware of the specialised sensory nerves that detect and transduce noxious stimuli. However, it was not until the late 19th Century that theories providing the formation for our current understanding of pain began to take shape.

The specificity theory of sensation which states that a given sense organ will respond to the stimuli it is adapted for, activity in a given nerve will always give rise to the same sensation, and that different parts of the central nervous system are responsible for specific sensations, was originated by Johannes Müller and Charles Bell (1842). Von Frey (1894) applied this theory to pain by suggesting that somatic sensory receptors existed for it thus leading to it

being established as a sensory experience. The concept of peripheral receptors for painful stimuli was readily accepted by Sherrington and Head who noted that in the cornea, where only the sensation of pain can be evoked, there is just one type of nerve-ending (more recently examined by Belmonte *et al.* (1991)). This led Sherrington to propose in 1906 that the detection of noxious stimuli be termed nociception, and that sensory neurones specifically sensitive to noxious stimuli be termed nociceptors. These nerves make connections with the dorsal horn of the spinal cord, from which subsets of neurones transmit signals to higher brain centres, including the reticular formation, thalamus and the cerebral cortex, in which conscious recognition of pain occurs (Basbaum and Jessell, 2000).

For pain to be perceived, Tillman *et al.* (1995) have shown that a certain discharge frequency must be reached in the nociceptive neurones. The amount of pain perceived for a given stimulus, however, is also dependent on physiological state. In the presence of inflammation both allodynia (the perception of pain due to a normally innocuous stimulus) and hyperalgesia (an increased sensation of pain for a given noxious stimulus) occur (Cervero and Laird, 1996). The role of hyperalgesia is to prevent further injury to an already damaged area of tissue. Hyperalgesia localised to the site of injury is termed primary hyperalgesia, whereas hyperalgesia in the surrounding uninjured area is termed secondary hyperalgesia (Lewis, 1935, 1942). Primary hyperalgesia is believed to be as a result of the sensitisation of nociceptors (Meyer and Campbell 1981, La Motte *et al.* 1982). In this respect nociceptors show a fundamental difference from other sensory receptors. Nociceptors, as with other sensory receptors, demonstrate adaptation—in this case to noxious stimuli that fail to produce actual tissue damage (Cesare and McNaughton, 1997). However, unlike other sensory receptors, they demonstrate the ability to be sensitised by mediators released when tissue is damaged. Inflammatory mediators include bradykinin (Beck and Handwerker, 1974), prostaglandins (Taiwo *et al.*, 1987), 5-hydroxytryptamine (5-HT), histamine (Coelho *et al.*,

1998), and nerve growth factor (NGF) (Amann *et al.*, 1996). Increased activity in the primary afferent neurone also contributes to inflammation (Lembeck and Holzer, 1979) via a phenomenon known as neurogenic inflammation in which neuropeptides such as calcitonin gene related peptide (CGRP) and substance P are released (Lynn, 1996) thus providing a positive feedback on sensitisation from the nociceptive nerve terminals themselves. Secondary hyperalgesia on the other hand is believed to be as a result of an augmentation of the response of spinal pain-signalling neurones to input from low-threshold mechanoreceptors (central sensitization) (Woolf, 1983).

1.2 Nociceptive neurones

Classification

In general, fine nerve fibres originating from medium- and small-diameter cell bodies in dorsal root ganglia (DRGs) are implicated in nociception—specifically thinly myelinated Aδ fibres, and slower conducting unmyelinated C fibres. The Aδ fibres are generally involved in mechanical nociception (Burgess and Perl, 1967) but some have been reported also to be excited by noxious heat (Iggo and Ogawa, 1971), whereas the C fibres are polymodal—responding to strong mechanical stimuli, noxious heat and irritant chemicals (Bessou and Perl, 1969). A-fibres have been shown to mediate the fast, pricking quality of pain whereas C fibres mediate the slower, burning quality of pain (for review see Stucky *et al.*, 2001). C fibres comprise around 70% of all nociceptors and histochemical studies of adult DRGs have shown that they can be split into two groups: a peptidergic group, containing substance P,

CGRP and expressing the TrkA receptor for NGF (Averill *et al.*, 1995); and a non-peptidergic group that express $P2X_3$ receptors (Bennet *et al.*, 1998) and can be labelled selectively with the isolectin B_4 (IB_4). In addition, there are some nociceptors—'silent' nociceptors—that only respond when sensitised by tissue injury (Schmidt *et al.*, 1995).

Excitation

In recent years the molecular basis underlying the transduction of noxious stimuli has been advanced as a result of the use of isolated sensory neurones in culture (Baccaglini and Hogan, 1983; Cesare and McNaughton, 1996) and the cloning of specific nociceptive genes (Akopian *et al.*, 1996). In general, a range of receptors for different noxious stimuli present in nociceptive neurones results in the polymodal nature of nociceptors. These receptors include: ATP-gated channels composed of a nociceptor specific $P2X_3$ subunit (Chen *et al.*, 1995; Lewis *et al.*, 1995; Cockayne *et al.*, 2000); $5HT_2$ (Todorovic and Anderson 1990) and $5HT_3$ receptors (Robertson and Bevan, 1991; Fozard, 1984); acid sensing ion channels (Waldmann *et al.*, 1997; Waldmann and Lazdunski, 1998); H_1 receptors for histamine (Tani *et al.*, 1990); the transient receptor potential vanilloid 1 cation channel (TRPV1; formerly known as the vanilloid receptor) involved in detecting capsaicin and noxious heat (Caterina *et al.*, 1997); and B_2 receptors which detect the production of bradykinin and thus the release of proteolytic enzymes from damaged cells (Burgess *et al.*, 1989; Mizumura *et al.*, 1990). Whereas some of the transducers are ligand-gated ion channels, H_1, $5HT_2$ and B_2 are G-protein coupled receptors and thus bring about their effects indirectly. B_2 receptors, via the production of 1,2-diacylglycerol (DAG) and activation of protein kinase C (PKC), depolarise neurones by opening a non-selective cation channel (Burgess *et al.*, 1989; McGehee and Oxford, 1991).

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The initial identification of this ion channel was followed by the identification of other ion channels which were also members of the TRP ion channel family and were found to cover the range of thermosensation from cold to hot (for review see Patapoutian *et al.*, 2003) as the thermosensation of different sub-populations of DRG neurones was identified. Patapoutian *et al.* (2003) dubbed these channels thermoTRPs as temperature alone could activate them. After TRPV1, the TRPV2 channel, which responds to temperatures greater than 52°C, was the next to be discovered (Caterina *et al.*, 1999). McKemy *et al.* (2002) cloned a TRP ion channel activated by menthol and cool (with a threshold of approximately 26°C). The range of TRP ion channels, as noted above, covers the range of temperatures from cold to hot: ANKTM1 (≤17°C), TRPM8 (≤25°C), TRPV4 (~27-42°C), TRPV3 (>33°C), TRPV1 (≥42°C), and TRPV2 (≥52°C) (See table 1 in Patapoutian *et al.*, 2003). The expression of these ion channels is not necessarily in mutually exclusive sub-populations of DRG neurones as is seen by the co-expression of TRPV1 with ANKTM1 (Story *et al.*, 2003). Although these ion channels share the characteristic in that they can be activated by the temperature alone, other characteristics differ e.g. in their response to repeated challenges: TRPV3 shows marked sensitisation (Peier *et al.*, 2002; Xu *et al.*, 2002) whereas TRPV4 shows desensitisation (Guler *et al.*, 2002). TRPV1, as well as showing slight sensitisation to repeated challenges (Jordt and Julius, 2002), can also be activated by a range of stimuli.

$5HT_2$ receptors depolarise dorsal root ganglion (DRG) neurones by reducing a resting K^+ conductance (Todorovic and Anderson, 1990). Thus noxious stimuli are transduced into a net inward current which can result in the depolarisation of the nociceptors, and formation of an action potential.

Of the transducers of noxious stimuli mentioned above, the ligand-gated ion channel TRPV1, which was cloned by Caterina *et al.* (1997), is one of the best understood. * It is activated by capsaicin (Caterina *et al.*, 1997), the pungent ingredient of chilli peppers, the endocannabinoid anandamide (Zygmunt *et al.*, 1999; Smart *et al.*, 2000), and noxious temperatures greater than 43°C (Tominaga *et al.*, 1998), the last matching the threshold of heat-evoked electrophysiological responses in cultured sensory neurones (Cesare and McNaughton, 1996). Both the capsaicin-evoked responses (Caterina *et al.*, 1997; Tominaga *et al.*, 1998) and the heat-activated currents (Tominaga *et al.*, 1998) can be modulated by protons. In mice lacking this receptor there are no responses to capsaicin and detection of noxious heat is impaired (Caterina *et al.*, 2000). As capsaicin sensitivity is a major trait of a large sub-population of nociceptive neurones, monitoring the activity of TRPV1 provides a suitable means of monitoring the responsiveness of nociceptive neurones. Indeed, double-labelling studies have shown that TRPV1 is expressed by both unmyelinated peptidergic and nonpeptidergic neurones in DRG (Tominaga *et al.*, 1998; Michael and Priestley, 1999). Michael and Priestley (1999) also found a population of TRPV1 positive neurones that did not belong to either of these groups. These very small DRG neurones, however, were found to represent just over 1% of the cells examined.

A range of ion channels, which can be subject to modulation, are involved in the excitatory state of nociceptive neurones. As in all neurones, voltage-gated sodium channels play an important role in excitability and action potential generation. Some DRG neurones, as well as expressing a tetrodotoxin-sensitive sodium current, also express a tetrodotoxin-resistant

(TTX-R) sodium current which is especially predominant in the capsaicin-sensitive subpopulation (Arbuckle and Docherty, 1995). Two ion channels have been found to underlie this TTX-R sodium current: $Na_v1.8$ (Akopian *et al.*, 1999), and $Na_v1.9$ (Tate *et al.*, 1998; Cummins *et al.*, 1999). These ion channels have been found to be important in regulating the excitability of DRG neurones (Cummins *et al.*, 1999) to noxious stimuli (Akopian *et al.*, 1999), and in inflammatory hyperalgesia (Khasar *et al.*, 1998; Akopian *et al.*, 1999).

I_A (a type of K^+ current) is one of the main factors regulating the maximum firing frequency of neurones (Connor and Stevens, 1971), and is expressed by small diameter capsaicin-sensitive neurones (Cardenas *et al.*, 1995). $I_{K(Ca)}$ 'small' conductance (SK) channels are responsible for a very prolonged afterhyperpolarisation (AHP) seen in a subpopulation of C neurones (Akins and McCleskey, 1993) which has a major influence on the ability of a neurone to fire repetitively in response to a maintained depolarisation (Weinreich and Wonderlin, 1987). This slow AHP is susceptible to inhibition by inflammatory mediators such as prostaglandins (Weinreich and Wonderlin, 1987) which results in the promotion of repetitive firing. I_h is a non-selective voltage-dependent channel activated by hyperpolarisation and is found in sensory neurones. Its potentiation may lead to more rapid repolarisation after an action potential and to facilitation of repetitive firing (Ingram and Williams, 1996). A range of voltage-gated calcium channels are found in sensory neurones, of which N- and P/Q-type channels in particular are important because of their role in mediating transmitter release (Reuter 1996; Santicioli *et al.*, 1992).

Sensitisation

As mentioned above sensitisation of nociceptive neurones underlies primary hyperalgesia. The principal situation in which this occurs is inflammation. Components of the "inflammatory soup" (which are chemical mediators released from primary sensory neurones and non-neuronal cells such as mast cells and neutrophils following tissue injury, Julius and Basbaum, 2001) can either sensitise nociceptors by directly interacting with ion channels or by mediating their effects indirectly through second-messenger signalling cascades. As discussed above, protons can directly modulate the responsiveness of TRPV1. 5HT (Cardenas *et al.*, 2001), tumour necrosis factor α (TNFα) (Junger and Sorkin, 2000), NGF (Shu and Mendell, 1999), histamine (Mizumura *et al.*, 1994), prostaglandins (England *et al.*, 1996) and bradykinin (Burgess *et al.*, 1989) all modulate the sensitivity of nociceptors through intracellular signalling mechanisms. One of the intracellular signalling pathways utilised involves the activation of PKC—this is the case with bradykinin (Burgess *et al.*, 1989) where the activation of PKC augments both the heat-evoked current in cultured DRG neurones via the epsilon isoform (Cesare and McNaughton, 1996; Cesare *et al.*, 1999a) and enhances the response to capsaicin in these neurones (Vellani *et al.*, 2001). As reviewed by Jaken (1996) there are at least 12 isoforms of PKC, which can be divided into 3 groups: conventional isoforms which are activated by calcium and phorbol esters (α, β_I, β_{II} and γ); novel isoforms which are activated by phorbol esters but are calcium independent (δ, ε, θ, η and μ); and atypical isoforms (ζ, λ and τ) which are calcium-independent and phorbol ester-insensitive. Five isoforms were found by Cesare *et al.* (1999a) in the cultured neonatal rat DRG neurones: PKC- β_I, β_{II}, δ, ε, and ζ. In embryonic (Olah *et al.*, 2002) and adult (Ma *et al.*, 2001) rat DRG neurones, however, the isoform PKC-α was also found.

The other major intracellular signalling pathway involved in the sensitisation of nociceptive neurones is the cyclic 3',5'-adenosine monophosphate/protein kinase A (cAMP/PKA) pathway. Prostaglandins (Hamprecht and Schultz 1973) and 5HT (Neufeld *et al.*, 1982) have both been shown to elevate intracellular cAMP in numerous tissues, and analogues of cAMP have been shown to produce hyperalgesia (Taiwo *et al.*, 1989).

Prostaglandin E_2 (PGE_2) as an inducer of primary hyperalgesia

Prostaglandins are known to be potent mediators of inflammation, and in tissues exhibiting an inflammatory response prostaglandins are elevated. The predominant prostaglandin involved in inflammation is PGE_2 (Willis, 1969; Piper and Vane, 1969) and one of its effects is the generation of hyperalgesia, which underlies the pain of inflammation. This has been established in a range of *in vivo* and *in situ* studies, including those showing that PGE_2 injection both increases the sensitivity of nociceptive neurones to noxious stimuli, including capsaicin, and recruits fibres that were insensitive before to the same noxious stimulus (Mense, 1981; Yanagisawa *et al.*, 1986; Taiwo *et al.*, 1989; Taiwo and Levine, 1991). The effect has been confirmed in cultured DRG neurones to be via a direct action which enhances their excitability and recruits neurones that did not respond to noxious stimuli before (Baccaglini and Hogan, 1983; Nicol and Cui, 1994; Cui and Nicol, 1995; Stucky *et al.*, 1996; Gold *et al.*, 1996a). The clinical importance of prostaglandin hyperalgesia is emphasised by the fact that the analgesic action of the potent non-steroidal anti-inflammatory drugs involves inhibition of the cyclo-oxygenase (COX) pathway (Smith & Willis, 1971) and thus inhibition of prostaglandin synthesis at peripheral sites of inflammation (Ferreira 1972). The PGE_2 evoked hyperalgesia has been shown to involve the activation of the cAMP/PKA pathway

(Taiwo *et al.*, 1989; Pitchford and Levine, 1991; Taiwo and Levine, 1991; Cui and Nicol, 1995) via the PGE_2 receptors EP3C and EP4 (Southall and Vasko, 2001). Recently, the molecular nature of this PGE_2 sensitisation has been elucidated. Gold *et al.* (1996a) and England *et al.* (1996) both showed that PGE_2 caused a modulation of the TTX-R sodium current present in nociceptive neurones. This current was both increased in magnitude, and its activation threshold was shifted in a hyperpolarised direction resulting in a general increase in excitability. Lopshire and Nicol (1998) have also shown that PGE_2 affects the capsaicin mediated current by increasing the activity of the TRPV1 ion channel (Caterina *et al.*, 1997). PGE_2 can also enhance the excitability of DRG neurones by inhibiting voltage-gated potassium currents (England *et al.*, 1996; Nicol *et al.*, 1997) and enhancing the I_h current (Ingram & Williams, 1996).

Given our current knowledge of the molecular basis of nociception and hyperalgesia, it is worth examining how it would be possible to attenuate these phenomena. One manner would be to use physiological 'antagonism' to attenuate the sensitisation of nociceptors and/or moderate their response. Recently the possibility of using a cannabinoid receptor agonist for this purpose has come to light.

1.3 Cannabinoid Receptors

Throughout history cannabis has been used in various cultures for its analgesic actions (Abel, 1979)—a fact that was brought to the attention of the Western medical establishment in the 19th Century (O'Shaugnessy, 1842; Reynolds, 1890). This analgesic action, as well as the

other neurobehavioural and physiological effects of cannabis, is mediated by the active components known as cannabinoids, of which Δ^9-tetrahydrocannabinol (Δ^9-THC) is the principal member (Gaoni and Mechoulam, 1964). The means by which cannabinoids brought about their effects in the body was originally believed to be by a cell membrane perturbing action (Lawrence and Gill, 1975; Hillard *et al.*, 1985). A series of reports, however, suggested the involvement of a G protein-coupled receptor, after the identification in brain and neural cell lines of a high affinity, stereoselective site for cannabinoids which mediated the inhibition of adenylate cyclase in a pertussis toxin-sensitive manner (Howlett and Fleming, 1984; Howlett, 1985; Howlett *et al.*, 1986; Devane *et al.*, 1988). These reports were soon followed by the cloning of the first cannabinoid receptor (CB_1) (Matsuda *et al.*, 1990) (mainly associated with neuronal tissue and the central nervous system (CNS)), and subsequently by the cloning of a second cannabinoid receptor (CB_2) which is mainly associated with non-neuronal tissue such as the immune system (Munro *et al.*, 1993). Recently, putative endogenous cannabinoids which are derivatives of arachidonic acid have been identified: arachidonyl ethanolamide (anandamide; AEA) (Devane *et al.*, 1992), 2-arachidonylglycerol (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), 2-arachidonyl glyceryl ether (Hanus *et al.*, 2001) and palmitoylethanolamide (PEA) (Facci *et al.*, 1995).

These discoveries resulted in the proposal that the activation of cannabinoid receptors in the CNS by either exogenous or endogenous cannabinoids produces antinociception (Yaksh, 1981; Lichtman and Martin, 1991; Martin *et al.*, 1993; Smith *et al.*, 1994; Meng *et al.*, 1998), and that endogenous cannabinoids have a role in tonically regulating pain thresholds in the CNS (Meng *et al.*, 1998). Recently, however, much interest has surrounded reports that cannabinoids can affect nociceptive processing by interacting with receptors in the periphery; an effect that is appealing when developing prospective therapies given the potential to separate the beneficial actions from the undesirable, psychoactive effects of cannabinoids

acting upon receptors in the brain (Degenhardt and Hall, 2002). These studies, discussed below, have ranged from *in vivo* behavioural experiments to localisation studies utilising radioligand binding, *in situ* hybridisation, and immunohistochemistry, to studies utilising cultured DRG neurones. Furthermore, although studies showing antinociceptive effects of cannabinoids may have physiological interest, antihyperalgesic effects are much more desirable if a future pharmaceutical is desired given the importance of inflammatory pain in clinical settings (e.g. postoperative pain). Such antihyperalgesic effects have been seen in behavioural studies on the peripheral actions of cannabinoids, in the context of various models of sensitisation.

In vivo studies

The carrageenan model of hyperalgesia, whereby the injection of this seaweed extract results in a time-dependent inflammation and subsequent thermal hyperalgesia, is a useful model for assessing anti-hyperalgesic and anti-inflammatory agents (Sammons *et al.*, 2000). The hyperalgesia produced by both peripheral mechanisms involving primary nociceptors and central mechanisms involving spinal cord neurones (Richardson *et al.*, 1998a; Richardson *et al.*, 1998b) was seen to be prevented in a dose-dependent manner by intraplantar and intrathecal administration of anandamide (an endogenous agonist of CB_1 receptors (Devane *et al.*, 1992)). The action of the endocannabinoid in both cases was linked, via CB_1 receptors, to an inhibition of the release of CGRP, an inflammatory neuropeptide released from primary afferent fibres—from central terminals with respect to the intrathecal administration, and from peripheral terminals with respect to the intraplantar administration. Interestingly, intrathecal administration of the same dose of anandamide in normal animals was not seen to have any

antinociceptive effect, thus suggesting that the attenuation acted on a factor involved in hyperalgesia but not normal nociception (Richardson *et al.*, 1998b). In the experiments utilising intrathecal administration, the antihyperalgesic effect was probably as a result of the prevention of afferent barrage. Afferent barrage is the high frequency stimulation of primary afferents that results in the sensitisation of dorsal horn neurones and thus secondary hyperalgesia. Anandamide, by acting presynaptically on the central terminals of primary afferents, could prevent this by inhibiting neuropeptide release. This was suggested to be either via the inhibition of calcium channels (Mackie and Hille, 1992; Caulfield and Brown, 1992) which would prevent exocytosis of the neuropeptides which function as transmitters at the dorsal horn, or by the opening of potassium channels (Deadwyler *et al.*, 1993; Henry and Chavkin, 1995) which would stabilise the pre-synaptic membrane potential.

In the experiments utilising intraplantar injection, the inhibition of hyperalgesia could be due to the prevention of neurogenic inflammation. This would likely be by the inhibition of neuropeptide release from peripheral terminals because of the location of the drug. Furthermore, in these experiments, anandamide was shown to be able to attenuate the maintenance of hyperalgesia. This possibly suggests an involvement of CB_1 receptors, expressed on peripheral nociceptors, in attenuating the sensitising effects of inflammatory mediators that are present after the induction of neurogenic inflammation. Thus, this activation of CB_1 receptors on peripheral terminals is probably inhibiting primary hyperalgesia directly, and secondary hyperalgesia indirectly.

The formalin model is another means of inducing hyperalgesia that has been used to elucidate how the activation of cannabinoid receptors interact with inflammatory hyperalgesia. There are two phases to the formalin model: the first phase involves the acute activation of nociceptive C fibres in response to the injection process itself, whereas the second phase involves the response to the sensitisation of the nociceptive pathway both at the

level of the primary afferent (peripheral sensitisation) and the level of the dorsal horn (central sensitisation) (Tjolsen *et al.*, 1992). Both Jaggar *et al.* (1998) and Calignano *et al.* (1998) found that the activation of CB_1 receptors or CB_2 receptors by a range of cannabinoid agonists, including anandamide, PEA, WIN 55,212-2 and HU210, attenuated the second phase of the behavioural responses to formalin injection thus suggesting an antihyperalgesic role mediated by the activation of these receptors. Although the actions of PEA via CB_2 receptors is most likely a peripheral mechanism given the location of these receptors on non-neural tissue, the actions of CB_1 agonists could be in either a central or peripheral location. Jaggar *et al.* (1998) used an intraperitoneal route of administration for anandamide and so the antihyperalgesic effect of anandamide could not be localised to either a central or peripheral effect. Calignano *et al.* (1998), however, showed that a peripherally active dose of anandamide given by the intraplantar route was much less effective when given by a systemic route (e.g. intravenously) and thus established a peripheral mechanism of action. Therefore at least some of the effect reported by Jaggar *et al.* (1998) may have been mediated by cannabinoid receptors in the periphery. Furthermore, Nackley *et al.* (2003) found that intraplantar injection of WIN 55,212-2 attenuated inflammatory nociception via peripheral cannabinoid receptors. Given the findings of the studies using carageenan injection, discussed above, anandamide may be affecting hyperalgesia by inhibiting neurogenic inflammation, which would prevent peripheral sensitisation and primary afferent barrage. A role, however, for a direct modulatory effect on the sensitisation of nociceptive neurones by inflammatory mediators cannot be ruled out.

A point of disagreement between two of the papers is the means by which PEA brings about its antihyperalgesic effect. Jaggar *et al.* (1998) suggest that PEA activates CB_2 receptors on mast cells to inhibit the release of inflammatory mediators (Facci *et al.*, 1995; Mazzari *et al.*, 1996) which otherwise could be expected to sensitise primary afferents. Calignano *et al.*

(1998), however, discounted this possibility as they failed to detect any effect on inflammatory oedema that overlapped with the effect on hyperalgesia. It is likely that the former scenario holds some truth, but with the recent potential detection of CB_2–like immunoreactivity on cultured DRG neurones (Ross *et al.*, 2001), a direct effect on peripheral afferents cannot now be ruled out.

Finally, another *in vivo* model of hyperalgesia uses capsaicin (the pungent extract of chilli peppers). Exposure of nociceptive neurones to capsaicin initially leads to the excitation of the neurone (resulting in the perception of pain and the local release of inflammatory mediators such as substance P and CGRP) (Winter *et al.*, 1995). This is followed by the development of both primary and secondary hyperalgesia (Baumann *et al.*, 1991; LaMotte *et al.*, 1992). Indeed, the exposure of human skin to capsaicin has been reported to result in hyperalgesia (Simone *et al.*, 1989). WIN 55,212-2 has been seen both to inhibit dose-dependently the nocifensive response produced by an injection of capsaicin, and to also block the development of mechanical and thermal hyperalgesia (Li *et al.*, 1999; Johanek *et al.*, 2001). Notably, no effect on basal withdrawal latencies to thermal stimuli was seen, thus reinforcing the theory that WIN 55,212-2 was antihyperalgesic and not antinociceptive. Because in this study the route of administration was intravenous, it is not possible to specify if the action was centrally- or peripherally-mediated. A peripheral site of action is, however, possible as the prevention of the release of inflammatory neuropeptides from the nociceptors (as discussed above) would both limit inflammatory sensitisation of the primary afferent fibres themselves (peripheral sensitisation), and aid in the reduction of primary afferent barrage, resulting in central sensitisation. Indeed, Ko and Woods (1999) also used a capsaicin model of hyperalgesia and found that Δ^9-THC *peripherally* inhibited, via CB_1 receptors, the development of thermal allodynia following the injection of capsaicin. As with the study by Li *et al.* (1999), no antinociceptive effect to a noxious thermal stimulus was seen in the absence of capsaicin, thus

reinforcing the antihyperalgesic nature of the effect of the cannabinoid. More recent reports, however, have shown that the activation of peripheral cannabinoid receptors can result in antinociception: Dogrul *et al.* (2003) found that WIN 55,212-2 applied topically to the tail of a mouse can have an antinociceptive effect in the tail-flick test via CB_1 receptors; Malan *et al.* (2001) found that the activation of CB_2 receptors had an antinociceptive action with respect to thermal stimuli; and Sokal *et al.* (2003) found that anandamide inhibited mechanically-evoked responses under inflammatory conditions via CB_2 receptors.

Various *in vivo* behavioural models have therefore shown that the activation of cannabinoid receptors in the periphery has an antihyperalgesic effect, as well as possibly an antinociceptive effect. An antihyperalgesic effect can be as a result of an inhibition of the release of inflammatory mediators as well as an attenuation of their effect on nociceptive neurones. Quartilho *et al.* (2003) found that the activation of peripheral CB_2 receptors inhibited inflammation and the associated hyperalgesia—a result borne out by Nackley *et al.* (2003) in a carrageenan model of inflammation. The mechanisms underlying the anti-inflammatory actions of endocannabinoids include attenuation of NGF-induced mast cell degranulation and of neutrophil accumulation (Rice *et al.*, 2002), as well as the attenuation of NO release by astrocytes (Molina-Holgado *et al.*, 2002), on which CB_1-like immunoreactivity has been detected (Salio *et al.*, 2002).

Because of the nature of behavioural studies it is difficult to ascertain all of the possible effects of cannabinoids on the neurones themselves in this respect. Such a problem would be solved by *in vitro* studies, but first it is worth discussing reports confirming the localisation of cannabinoid receptors to nociceptive neurones.

Localisation studies

Although the *in vivo* behavioural studies strongly suggest that cannabinoids have a peripheral site of action with respect to the modulation of nociceptive neurones, and that actions via both CB_1 and CB_2 receptors are important, they do not confirm the precise location of the receptors in the periphery that are important for these actions. A range of localisation studies has been carried out to ascertain if primary afferent fibres involved in nociception express cannabinoid receptors, and to see which subpopulations of nociceptors are important. Richardson *et al.* (1998a), utilising radioligand binding studies, noted that cannabinoid receptors could be expressed on primary afferent fibres. Furthermore, the actions of cannabinoids in inhibiting the release of neuropeptides from the central terminals of primary afferents (Richardson *et al.*, 1998a) would suggest their localisation on these primary afferents. Indeed, Hohmann and Herkenham (1998) did find a reliable suppression of cannabinoid receptor binding sites following long term neonatal capsaicin treatment (known to destroy nociceptive primary afferents), providing an anatomical basis for this action. The suppression of binding, however, was only moderate suggesting that the majority of cannabinoid receptors in the superficial dorsal horn did not lie presynaptically on the central terminals of these capsaicin sensitive nociceptors. A further *in vitro* receptor binding study utilising dorsal rhizotomy demonstrated that 50% of cannabinoid binding sites at the level of the spinal cord reside on the central terminals of primary afferents (Hohmann *et al.*, 1999). To ascertain on which neuronal subpopulations of DRGs cannabinoid receptors are located, a double-label *in situ* hybridisation study was carried out (Hohmann and Herkenham, 1999b). CB_1 messenger RNA (mRNA) was detected in DRG neurones thus showing that DRG neurones can express cannabinoid receptors. Furthermore, some overlap with neurones expressing neuropeptide markers indicative of nociceptive neurones (i.e. CGRP and substance

P) was demonstrated, again providing evidence for an anatomical substrate underlying the cannabinoid-mediated inhibition of CGRP release from both peripheral and central terminals of nociceptive neurones (Richardson *et al.*, 1998a; Richardson *et al.*, 1998b). Also CB_1 mRNA was restricted to 10-15% of DRG neurones, the majority of which did not express these peptide markers and were of a large diameter (suggesting a localisation to Aβ and Aδ fibres). A further study using *in vitro* receptor binding and sciatic nerve ligation demonstrated that these cannabinoid binding sites did undergo transport to the peripheral terminals of the primary afferent nerves (Hohmann and Herkenham, 1999a). This study also found no evidence that CB_2 receptor mRNA was synthesised by DRG neurones.

Despite these studies, Farquhar-Smith *et al.* (2000), utilising an antibody raised against part of the C-terminal of the CB_1 receptor, found in the dorsal horn that, whereas there was localisation in the same lamina of CB_1-like immunopositive neurones with neurones expressing markers of primary nociceptive neurones (IB_4, CGRP and TRPV1), there was little co-localisation at fibre level. From this they suggested that CB_1 receptors were expressed predominantly on axons of intrinsic spinal cord neurones. Although this could perhaps explain the actions of cannabinoids in moderating nociceptive responses at the spinal level (Richardson *et al.*, 1998b), the argument does not appear to take into account the inhibition of release of CGRP from peripheral terminals of primary afferents (Richardson *et al.*, 1998a) nor do the conclusions correspond with the presence of CB_1 receptors in at least some nociceptive neuronal terminals (as discussed above). Furthermore, they found a reduction of less than 5% of the CB_1-like immunoreactivity following a dorsal rhizotomy adequate to eliminate IB_4 staining. It could be suggested that expression on spinal interneurones and primary afferent fibres are both found, given that the reduction in cannabinoid radioligand binding following dorsal rhizotomy is only 50% (Hohmann *et al.*, 1999). Explanations for the inconsistency with other reports could be that another subtype of receptor is present in the nociceptive

neurones, or that there were difficulties with this antibody. Further studies utilising two different antibodies raised against the N-terminal of CB_1 receptors (Sanudo-Pena *et al.*, 1999; Ahluwalia *et al.*, 2000; Ross *et al.*, 2001) indeed suggest that there may be a difficulty with the C-terminal targeted antibody. Sanudo-Pena *et al.* (1999) found that virtually all cells in DRGs (notably including smaller DRG cells) were labelled, and that the presence of CB_1-like immunoreactivity in the dorsal root and the proximal nerve suggested transport of receptors to both peripheral and central terminals of primary afferent neurones (thus underlining the functional studies discussed above). Furthermore, immunohistochemical studies on cultured dorsal root ganglion neurones showed that more than half of the neurones which belonged to the small soma size subpopulation showed CB_1-like immunoreactivity (Ahluwalia *et al.*, 2000), and that as well as expression on cell bodies, immunolabelling was evident on the processes of the cultured neurones (Ross *et al.*, 2001). Ahluwalia *et al.* (2000) also showed that almost all TRPV1-like immunopositive neurones (indicative of polymodal, capsaicin sensitive, nociceptive neurones) showed CB_1-like immunoreactivity. Interestingly, about 82% of CB_1-like immunopositive neurones showed TRPV1-like immunoreactivity, thus providing a correlation with previous studies showing that CB_1 receptors are located on sensory neurones other than those involved in nociception (Hohmann and Herkenham, 1998; Hohmann and Herkenham, 1999b). Suggestions that altered expression levels *in vitro* were a confounding factor in this study were rebutted by the observation that the extent of the expression of TRPV1-like immunoreactivity was similar to that found in intact DRGs. The most recent instalment in the examination of CB_1 receptor localisation shows that in rat DRG no more than 20% of CB_1-expressing cells co-stained for TRPV1 and that only about 7% of TRPV1 expressing cells were found to express CB_1 mRNA (Bridges *et al.*, 2003). Furthermore, Bridges *et al.* (2003) found that the majority of the 25% of DRG neurones that

expressed CB_1 receptors were myelinated A-fibre neurones, which links back to the findings by Hohmann and Herkenham (1999b).

An interesting finding from Ross *et al.* (2001) was that immunoreactivity to an antibody raised against the CB_2 receptor was found in cells within DRG cultures. Although this could be as a result of expression on non-neuronal cells in the culture, F-11 cells (a DRG x neuroblastoma hybridoma) used in the same study were also found to express CB_2 receptors. Thus studies utilising antibodies against CB_2 receptors on acutely isolated tissue may be worthwhile, though a functional effect has not been found yet given that SR144528 (a CB_2 antagonist) did not attenuate the actions of 100 nM WIN 55,212-2 (Ross *et al.*, 2001).

Therefore, although there has been some debate as to the extent and nature of the location of CB_1 receptors in primary afferent neurones, on the whole most studies show that at least some neurones likely to be nociceptors express CB_1 receptors (Hohmann and Herkenham, 1998; Sanudo-Pena *et al.*, 1999; Hohmann *et al.*, 1999; Hohmann and Herkenham, 1999a; Hohmann and Herkenham, 1999b; Ahluwalia *et al.*, 2000; Ross *et al.*, 2001; Bridges *et al.*, 2003). Although localisation studies help to provide an anatomical basis for the peripheral actions of cannabinoids, only functional studies will enable the extent of the actions of cannabinoids on nociceptive neurones to be fully understood. Therefore, it is appropriate to examine how cannabinoids modulate nociceptive neurones on a cellular basis. Thus *in vitro* studies using cultured DRG neurones are a suitable model as a subset of these neurones are known to express the characteristics of nociceptors *in vivo* (Baccaglini and Hogan, 1983; Gold *et al.*, 1996b).

In vitro culture studies

Because of the difficulty in dissecting out primary afferent fibres themselves, *in vitro* studies utilise cultures of the isolated dorsal root ganglion neurone cell bodies that give rise to these primary afferent fibres. Cultured nerve cell bodies are found to express receptors known to exist at both peripheral and central terminals of the primary sensory neurone. Therefore actions of cannabinoid receptor ligands on cultured DRG neurones may reflect events that occur centrally and/or peripherally. Utilising such a model, Ross *et al.* (2001) found that both anandamide and WIN 55,212-2 inhibited N-type voltage-gated calcium channels via CB_1 receptors (as seen by sensitivity to the CB_1 antagonist SR141716A). Furthermore, this inhibition was pertussis toxin-sensitive suggesting the involvement of $G_{i/o}$ proteins which have been reported before to mediate cannabinoid receptor effects (Howlett *et al.*, 1986). Such an inhibitory action could underlie previous studies that suggest that inhibition of neuropeptide release from the terminals of primary nociceptive neurones is important in the antihyperalgesic role of cannabinoids (Richardson *et al.*, 1998a; Richardson *et al.*, 1998b). Surprisingly, 100 nM WIN 55,212-2 did not noticeably inhibit forskolin-stimulated cAMP production (Ross *et al.*, 2001), which contrasts with other reports of cannabinoids inhibiting the production of cAMP (Howlett and Fleming, 1984; Howlett, 1985; Jung *et al.*, 1997). It was suggested though that the inhibition of forskolin-stimulated cAMP production in these cells might be too small to be measured experimentally. Interestingly, Jung *et al.* (1997) found that higher concentrations of WIN 55,212-2 were needed to inhibit forskolin-stimulated cAMP production than cAMP production stimulated by a receptor agonist (isoproterenol) thus suggesting that the effects of WIN 55,212-2 may have been counteracted by forskolin in the study by Ross *et al.* (2001). Even a small inhibition of cAMP may be functionally significant given the amplification of the cAMP cascade. Also noted in

the study by Ross *et al.* (2001) was that WIN 55,212-2 had an effect in all of the neurones tested.

In another study, Millns *et al.* (2001) showed recently that HU210 inhibited capsaicin-evoked calcium responses in cultured DRG neurones via CB_1 receptors. Not only does this support the existence of functional cannabinoid receptors in DRG neurones, but it also reinforces their presence in polymodal nociceptive neurones. This result was supported in the more recent study by Ahluwalia *et al.* (2003) where anandamide was shown to inhibit capsaicin-evoked CGRP release. Tognetto *et al.* (2001) also found that the activation of cannabinoid receptors on cultured dorsal root ganglion neurones could inhibit an increase in intracellular calcium concentration, and neuropeptide release, evoked by electric field stimulation. This could be due to the inhibition of voltage-gated calcium channels. A more recent report by Khasabova *et al.* (2002), however, found that while the activation of CB_1 receptors inhibited the response of intermediate diameter neurones to K^+, these neurones rarely responded to capsaicin. Furthermore, in those neurones most likely to respond to capsaicin, small-diameter neurones, there was generally no effect despite the presence of CB_1 receptor immunoreactivity.

Therefore, the evidence suggests that, on balance, functional cannabinoid receptors do exist in nociceptive neurones and this localisation is important in their modulatory actions, of which the inhibition of neuropeptide release via inhibiting N-type calcium channels is an important aspect. Although Ross *et al.* (2001) concluded that the cannabinoid receptor-mediated inhibition of the calcium channels was probably as a result of a direct action of the G protein upon the channels themselves, cannabinoids could also mediate their antihyperalgesic actions by other intracellular means. One potential route is via their inhibitory actions on adenylate cyclase (Howlett and Fleming, 1984; Howlett, 1985; Howlett

et al., 1986), the activation of which is known to mediate the peripheral sensitisation of nociceptors by inflammatory mediators, leading to primary hyperalgesia.

Aim

Given that opioids are known to bind to and activate receptors coupled to the inhibition of adenylate cyclase (Minami and Satoh, 1995), and that they too have a peripheral dimension to their control of nociception (Stein, 1995), similar mechanisms of action for cannabinoids in the periphery were considered. As the activation of μ opioid receptors has been seen to prevent the increase in peak TTX-R sodium current induced by PGE_2 (Gold and Levine, 1996), it was decided to investigate whether cannabinoids modulate the PGE_2 sensitisation of nociceptive neurones. This effect was examined by utilising capsaicin to evoke a change in intracellular calcium levels in the subset of cultured DRG neurones that express the TRPV1 receptor, which include nociceptive neurones. In addition to this, the effect of activating another more recently discovered family of G-protein coupled receptors (GPCRs), the Sensory Neurone Specific Receptors (SNSRs), was investigated.

1.4 Sensory Neurone Specific Receptors (SNSRs)

Cloning of the receptors

The novel family of GPCRs called Sensory Neurone Specific Receptors (SNSRs), also known as *Mas*-related gene (MRG) receptors, were identified independently by two groups

(Dong *et al.*, 2001; Lembo *et al.*, 2002). Both studies utilised cloning as a means of identifying novel GPCRs that were specific for nociceptive neurones. Cloning involves digesting deoxyribonucleic acid (DNA) from a selected source using restriction enzymes and then inserting it into a vector which, in turn, is inserted into a host. A range of vectors can be employed with plasmids being the principle ones. If it is necessary to clone large pieces of genomic DNA then the Bacterial Artificial Chromosome, which is a system based upon the single-copy plasmid F factor from *Escherichia coli*, can be employed (Shizuya *et al.*, 1992). In order to make it easier to isolate expressed genes (given that genomic DNA includes non-transcribed sections), messenger ribonucleic acid (mRNA) can be reverse transcribed into complementary DNA (cDNA) and this can be inserted into a vector. Once vectors containing the DNA from a source of interest are inserted into hosts such as *E. coli* a library is formed which can then be utilised to study the selected DNA (for more information, see Howe, 1995).

Dong *et al.* (2001) utilised a mutant phenotype of mouse where trkA positive neurones (which include a subpopulation of nociceptive neurones) failed to be generated. By identifying the cDNA present in a library generated from wild-type neurones, that was absent in this mutant population, this enabled genes specifically expressed in these neurones to be identified. One of the previously unknown genes was for a GPCR with homology for the *Mas* gene family, which was named *mrgA1* (*Mas-related gene A1*). Additional members of this family were found to be expressed by murine DRG by screening cDNA and BAC libraries (*mrgsA2-8)*, and by searching gene databases using mrgA1 as the query sequence (additional members of the *mrgA* subfamily, and two closely related subfamilies, *mrgB* and *mrgC*).

Lembo *et al.* (2002) identified a novel GPCR in rat (rSNSR) after using a cDNA library isolated from a primary culture of rat DRG. Subsequently, they identified six novel GPCRs (called hSNSRs) from the human genomic library using the sequence for rSNSR as a probe. These GPCRs were noted to have a close relationship with the MRGs.

Given the identification of what could be the same receptor family by two different groups, there is some confusion regarding the receptor nomenclature. Until this is officially resolved, there will be difficulty in knowing which is the more correct nomenclature to use, however, for the purposes of this thesis SNSR has been adopted. For the hSNSRs identified by Lembo *et al.* (2002), the following correlations were noted: SNSR1 is equivalent to hMRGX3 (with SNSR2 as its closest relative); SNSR4 is equivalent to hMRGX1 (with SNSR3 as its closest relative); SNSR6 is equivalent to hMRGX4 (with SNSR5 as its closest relative).

Also, the number of members of this GPCR family appears to be species dependent with Zylka *et al.* (2003) noting that in rat sensory ganglia there were a core subset of approximately four different genes (*MrgA, MrgB, MrgC and MrgD).*

Localisation

The name SNSRs derives from their very specific localisation to sensory neurones (Lembo *et al.*, 2002). Lembo *et al.* (2002) reported that the expression of SNSR mRNA was confined to a subset of small-diameter neurones in rat DRG and trigeminal ganglia—notably 56% of neurones expressing SNSR mRNA displayed TRPV1-like immunoreactivity. This suggests that SNSRs are expressed by a subset of nociceptive neurones. This was further suggested when *MrgC11*, another name for the rat ortholog, was found in small diameter, IB_4-positive nociceptive neurones (Han *et al.*, 2002). Interestingly, Dong *et al.* (2001) found that *mrgA*s and *mrgD*, the genes encoding the GPCRs, were expressed differently in subsets of murine sensory neurones, and were generally expressed in neurones with the trkA receptor for NGF but not TRPV1. A recent study by Zylka *et al.* (2003) examined the *Mrg* genes found in rats, and found that unlike mice, they had just a single *MrgA* and *MrgC* gene. In fact in rat DRG it

was found that there were principally four different genes (*MrgA*, *MrgB*, *MrgC* and *MrgD*), defining four different neuronal subpopulations (rMrgA & rMrgD, rMrgB4 & rMrgB5, rMrgC, and rMrgC & rMrgB4) all of which bound IB_4 and expressed the c-Ret receptor for GDNF. All Mrg^+ neurones were found to express $P2X_3$ receptors in the rat and all $rMrgD^+$ cells were found to express TRPV1. rMrgC, also defined as rSNSR1, was found to be present at both peripheral and central terminals of DRG by Grazzini *et al.* (2004). When these reports are taken together, SNSRs (or MRG receptors) appear to be expressed in a subset of small diameter, nociceptive neurones, the exact characteristics of which show subtle differences in alternate species. This localisation potentially implicates them in modulating nociceptive processing.

Agonists and receptors

Given the identification of these novel GPCRs, agonists which would activate these receptors were sought. After screening HEK293 cells stably expressing hSNSR3 or hSNSR4 using a calcium-mobilization assay, Lembo *et al.* (2002) found that bovine adrenal medulla peptide 22 (BAM 22) was the most potent agonist. However, because the classical opioid YGGFM (Met-enkephalin) motif is present in BAM 22, BAM (8-22) was found to be the best selective ligand. Han *et al.* (2002) found that BAM 22 was able to activate mouse MrgA1 weakly and MrgC11 potently. A more recent report from Grazzini *et al.* (2004) examined rat SNSR1 and found that the most potent agonist for this receptor was γ2-MSH. BAM (8-22) was also a full agonist at this receptor but its potency was poor compared with that at hSNSRs. Han *et al.* (2002) found that γ2-MSH was also a potent ligand for mouse MrgC11, thus helping to confirm its status as the ortholog of rat SNSR1.

In general it was found that Mrg receptors could be activated by RF-amide related peptides (Han *et al.*, 2002; Dong *et al.*, 2001), of which BAM 22 is a member, and pain-modulating opioid peptides such as BAM 22 (derived from proenkephalin) and γ2-MSH (derived from proopiomelanocortin) (Choi and Lahn, 2003). Han *et al.* (2002) suggested that post-processing of the RF amide peptides may determine Mrg receptor-ligand specificity. Such a concept fits well with the report by Choi and Lahn (2003) that the extracellular domains of the Mrg receptor family display evidence of strong positive selection thus permitting the mosaic of Mrg expression by nociceptive neurones to engender a wide range of sensitivity.

The endogenous agonist of these receptors is unknown, though Zhang *et al.* (1999) found that BAM (8-22) occurred endogenously as a peptidase cleavage product of proenkephalinA-derived peptide E. The precursor of γ2-MSH, POMC, has been detected in rat spinal cord, DRG and skin. Thus there is evidence that both BAM (8-22) and γ2-MSH could be potential endogenous ligands for SNSRs. As for the functional significance of these receptors only rat SNSR1 has been shown to be of actual functional significance (see below).

As for the coupling of these receptors *in situ*, Han *et al.* (2002) demonstrated that both MrgA1 and MrgC11 receptors are coupled to the $G\alpha_{q/11}$ signaling pathway in mouse embryonic fibroblasts by utilising Gα protein knockout mice. This does not, however, deal with whether this is the case in neurones, and so this remains to be determined.

In vivo studies

The role of these receptors with respect to nociception was partially elucidated by Cao *et al.* (2003) when in a study examining the rat spinal nociceptive flexor reflex they showed that the activation of SNSRs by the application of BAM (8-22) resulted in enhanced touch, pinch and heat responses. Grazzini *et al.* (2004) also found that the activation of rat SNSR1 produced

spontaneous pain behaviour, heat hyperalgesia and mechanical allodynia when injected intradermally, and heat hyperalgesia when given intrathecally.

Aim

The reports noted above do not examine either the real role of SNSRs at the cellular level in sensory neurones or the signalling pathways involved with SNSRs in real neurones. Given the pronociceptive effect of SNSR activation seen *in vivo*, and the localisation on neurones expressing TRPV1, it was decided to examine if the activation of SNSRs resulted in a modulation of the excitability of nociceptive neurones, and also if this affected TRPV1 itself. The possibility of examining the signalling pathway involved in real neurones, if an effect was seen, could then be possible.

Hypotheses to be tested

1) The activation of cannabinoid receptors on nociceptive neurones attenuates the sensitisation of these neurones by PGE_2.
2) The activation of SNSRs:-
    a. enhances the excitability of nociceptive neurones,
    b. activates an intracellular signalling pathway involving kinases, and
    c. modulates TRPV1.

Chapter 2

Materials and Methods

2.1 Primary culture of dorsal root ganglion neurones

Dissection

Neonatal Wistar rats aged 3 to 7 days old were sacrificed by cervical dislocation followed by decapitation. A dorsal midline incision through the skin was then made to reveal the spinal column. Next, another midline incision was made through the dorsal wall of the spinal column to reveal the spinal cord. The spinal cord was removed, and overhanging vertebrae were trimmed back slightly to provide a clear view of the ventral part of the spinal column. The dorsal root ganglia (DRGs), which are found between the vertebrae on both sides of the spinal column towards the ventral surface, were then harvested using ultra-fine forceps into 2 ml Dulbecco's Phosphate Buffered Saline (DPBS-1; Invitrogen; Cat No. 14040) in a 35 mm petri dish. Generally, 40 to 50 DRGs were harvested from one animal. The DRGs were then cleaned individually of nerve trunks and obvious connective tissue.

Dissociation

DRGs were transferred into 2 ml Medium-1 (see Table 2.1 for media formulations) containing 0.25% collagenase (type IV; Worthington). DRGs were incubated in this solution for 1 hour at 37°C, 5% CO_2, and were then washed twice with 10 ml Medium-2 before being triturated in 2 ml Medium-2; first using a 0.6 mm diameter needle, and then a 0.5 mm diameter needle until the DRGs had been visibly broken up. The dorsal root ganglion (DRG) cell suspension was washed through a 40 μm nylon cell strainer (BD Biosciences) with 8 ml Medium-2, before being centrifuged for 10 minutes at 1000 rpm. This filtration step is

expected to increase the percentage of cultured dorsal root ganglion (DRG) neurones expressing TRPV1, as these are at the smaller end of the size range (Michael and Priestley, 1999).

Plating on glass coverslips for calcium imaging and immunocytochemistry

The DRG cell pellet was resuspended in 1 ml Medium-3, and an approximate count of DRG neurones present in the suspension was then carried out using a haemocytometer. The concentration of neurones in the suspension was altered to approximately $5x10^4$ neurones ml^{-1}. 0.5 ml of this suspension were plated onto glass coverslips (13 mm diameter, thickness 1; BDH) previously coated for at least 30 minutes with 4.167 µg poly-L-lysine hydrobromide (cell culture grade; Sigma) and overnight with 2.5 µg laminin (BD Biosciences). Coverslips were kept at 37°C, 5% CO_2, and after 48 hours the Medium-3 was removed and replaced with 0.5 ml Medium-4.

Plating on 35 mm petri dishes for electrophysiology

The DRG cell pellet was resuspended in 0.5 ml Medium-3, and an approximate count of DRG neurones present in the suspension was then carried out using a haemocytometer. The concentration of neurones in the suspension was altered to approximately $1.33x10^5$ neurones ml^{-1}. 30 µl of this suspension was added to the centre of 35 mm petri dishes previously coated for at least 30 minutes with 8.334 µg poly-L-lysine hydrobromide. The petri dishes were kept at 37°C, 5% CO_2 for 30 minutes before 2 ml Medium-3 was added to each dish and they were

then returned to 37°C, 5% CO_2. After 48 hours, the Medium-3 was removed and replaced with 2 ml Medium-4.

Media Formulations

Media	**Ingredients**
Medium-1	Dulbecco's Modified Eagle Medium (high glucose; Cat No. 41966) with 1% penicillin-streptomycin (Cat No. 15070) and 2 mM L-glutamine; all from Invitrogen
Medium-2	Medium-1 with 10% fetal bovine serum (FBS; heat inactivated; Cat No. 10108; Invitrogen)
Medium-3	Medium-2 with 50 ng ml^{-1} nerve growth factor (NGF; murine, 7S; Promega) and 1.25 µg ml^{-1} cytosine 1-β-D-arabinofuranoside (Ara-C; Sigma)
Medium-4	Medium-1 with 1% N-2 supplement (Invitrogen), 50 ng ml^{-1} NGF and 0.5 µg ml^{-1} Ara-C

Table 2.1 Details of various media formulations employed

2.2 Calcium Imaging

Fluo-4 loading

Neonatal rat DRG neurones, cultured for 3 days on glass coverslips, were incubated in Medium-1 containing 8 μg ml^{-1} Fluo-4 AM (Molecular Probes) for 30 to 45 minutes at 37°C, 5% CO_2. Fluo-4 AM is a cell permeant ester form of Fluo-4 that is cleaved following uptake into the cell by intracellular esterases to form Fluo-4 that is cell impermeant. The intensity of excited emission of Fluo-4 increases when it has Ca^{2+} bound and it can thus be used to measure the intracellular calcium concentration. At room temperature, based upon assays carried out by Molecular Probes, the dissociation constant of Fluo-4 for calcium (K_d) is 345 nM, the absorption maximum is 494 nm, and the emission maximum is 516 nm. The free intracellular calcium concentration is related to the fluorescence by the following formula (Free intracellular calcium concentration = $Kd(F-F_{min})/(F_{max}-F)$). In the current study, however, it was not necessary to calculate the intracellular calcium concentration as a comparison of the normalised fluorescence was adequate.

Imaging of neurones

Coverslips were loaded into a confocal flow chamber (Figure 2.1; after McNaughton *et al.* (1990)), which was then filled with Hank's balanced salt solution (HBSS in mM: 140 NaCl, 4 KCl, 10 HEPES, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 glucose; pH 7.4 with NaOH). The level of Ca^{2+} present in the neurones was determined by using a Nikon Eclipse TE300 microscope, with a 20x, water-immersion Plan Fluor objective, connected to a Bio-Rad micro-Radiance Confocal

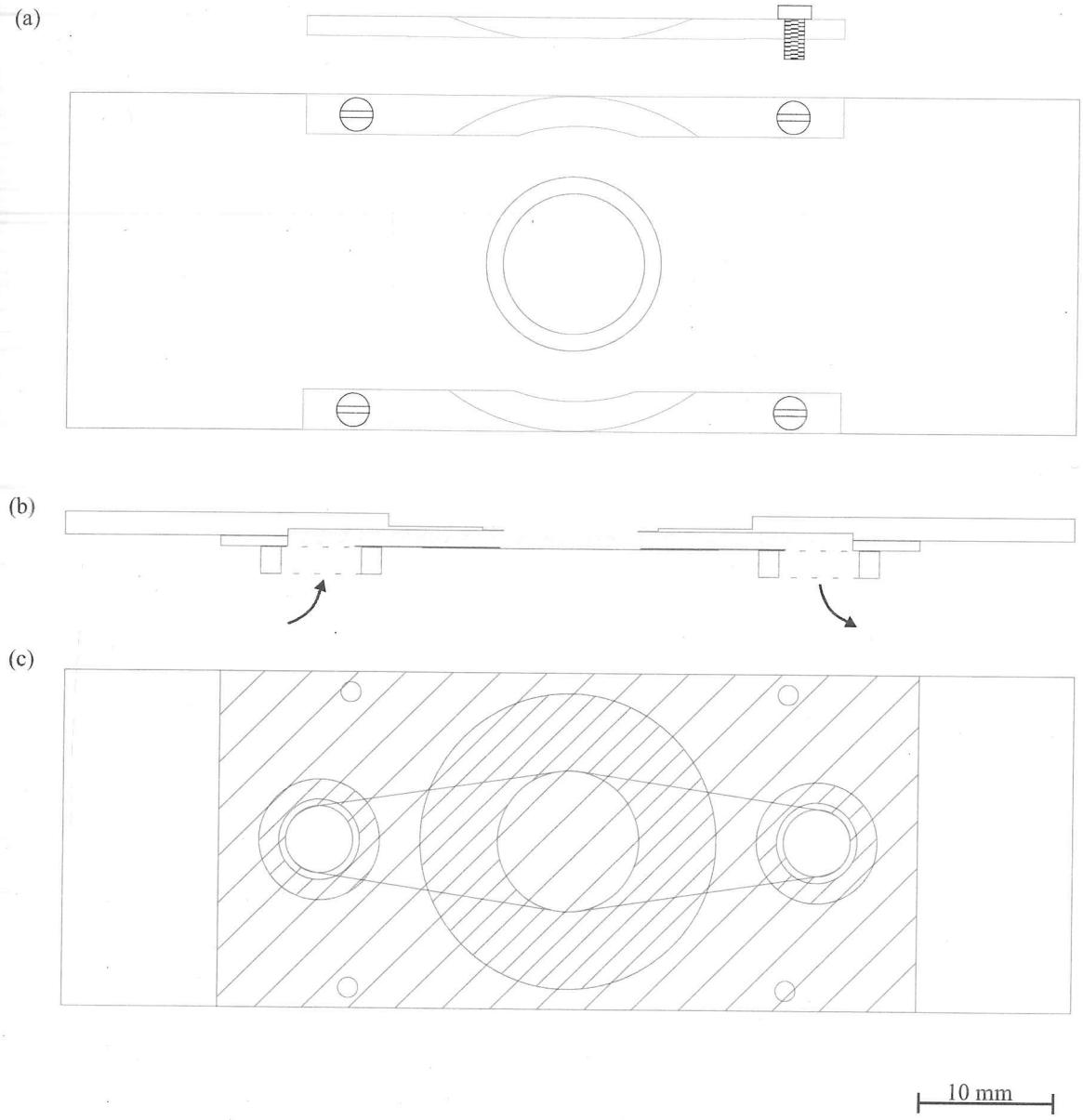

Figure 2.1 Technical diagram of the confocal flow chamber

The confocal flow chamber viewed from the top (a), in a lengthways bisection (b) and from the bottom (c). The main Duralumin component (red) contains a central recess into which the coverslip is placed cell-side down. The coverslip is retained in place by a Duralumin lid (yellow) which is secured by Duralumin brackets. The solution containing part of the chamber (light grey; approximate volume 0.4ml) is bounded on the other side by perspex (blue), with a 22mm diameter coverslip (BDH; borosilicate glass; thickness number 1) (green) completing the enclosure. Two pieces of silicon tubing (green) surround the inflow and outflow (indicated by arrows). All of these components are held together with silicon sealant.

Scanning system (model MRAG2) to detect the emission from Fluo-4, which was excited by an air-cooled argon/ion laser filtered to produce a beam of 488 nm. Thirty percent of the excitation light was reflected down to the sample using an 80:30 beam splitter that relies on the polarisation of light to distinguish laser light from light returning from the sample. Subsequently, eighty percent of emitted light passed through the 80:30 beam splitter to be completely channelled to a single photomultiplier before which it was filtered through a 500 nm long-pass filter to remove any excitation light. Laser Sharp software (BioRad; version 3.2, with Time Course attachment) was used to record fluorescence and transmission images (with a Kalman filter, n=2) every 3 seconds, and these were saved for later analysis. All experiments were carried out at 21±1°C.

The confocal flow chamber was designed to allow the perfusion of the cultured DRG neurones on the coverslip while calcium imaging was carried out using the confocal scanning system discussed above. The solution-containing part of the chamber was designed to allow laminar flow in one direction. An enclosed chamber design with open ends was used, as this prevented the chamber running dry while limiting the presence of bubbles in the chamber. A chamber volume of approximately 0.4 ml was selected as this permitted a rapid change of solution in the chamber (i.e. with a flow rate of 3.5 ml min^{-1} the solution in the chamber was completely changed in just under 7 seconds).

Time course

The confocal flow chamber was perfused with HBSS or drug treatments at approximately 3.5 ml min^{-1} by means of a gravity perfusion system. When test solutions were being added, the perfusion of HBSS or drug treatment was stopped; this was resumed after the addition was

completed. Test solutions were added manually using a 2 ml syringe, and this flow replaced the HBSS flow.

Neurones were exposed to HBSS and 0.01 or 0.02% DMSO (dimethyl sulfoxide; Sigma) for 15 seconds each in order to test for the effects of solution addition, and the effects of the vehicle, respectively. This was followed by a 15 second exposure to a 25 mM KCl solution (in mM: 119 NaCl, 25 KCl, 10 HEPES, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 glucose; pH 7.4 with NaOH) in order to confirm the identity of neurones. 100 nM capsaicin (Calbiochem; 5 mM stock solution in DMSO) was then applied for 15 seconds, every four minutes, to activate the transient receptor potential vanilloid 1 (TRPV1) cation channels. Drug treatment solutions were applied for a specified length of time before the sixth capsaicin addition (which also contained the drug treatment). Four minutes after the eleventh capsaicin addition, 100 μM α,β-methyleneadenosine 5'-triphosphate lithium salt (α,β-MeATP; Sigma) was applied for 15 seconds to identify those neurones expressing $P2X_3$ receptors. On the completion of the protocol, the perfusion of HBSS was stopped and 10 μM ionomycin (calcium salt; Sigma; in (in mM) 30 $CaCl_2$, 125 KCl, 10 HEPES; pH 7.4 with NaOH) was added in order to provide the maximum fluorescence (F_{max}).

Analysis

The pre-recorded fluorescence images were analysed using either Laser Sharp software, with Time Course attachment, or LaserPix (version 4.0, BioRad). Regions believed to contain individual DRG neurones were selected and the level of fluorescence (F) in each of these regions was recorded throughout the time course. The dark image value for a region (which is the signal collected when the laser shutter is closed and thus represents the dark

noise of the photomultiplier of the confocal system) was subtracted from all of the readings for that region. The readings were then standardised by dividing by the maximum value recorded for that region (F_{max}) to generate values in F/F_{max}. Figure 2.2 shows a plot of F/F_{max} against time for one such region of interest.

Because of the great number of regions of interest handled and the variability in the responses of different neurones, it was decided to develop an automated system capable of objectively handling the data to prevent any bias in the analysis. A spreadsheet (Microsoft Office Excel 2000; Microsoft Corporation) was designed to assess the characteristics of each region of interest by checking if the data for the region met a range of criteria which indicated that it covered a healthy neurone that repeatedly responded to capsaicin, showed negligible vehicle artefact (i.e. a response to the addition of the solvent itself), and had loaded well with the Fluo-4 AM. By ensuring that a region of interest fell within this range of criteria, it was possible to minimise the noise associated with the variability of the responses. The criteria (where peaks are always as compared to the averaged baseline for the 30 seconds preceding the addition of the test solution) were as follows:-

a) **Dye loss**: that the highest reading of fluorescence was generated after the addition of ionomycin. This controls for rapid dye loss by leaking.

b) **Cell is a neurone**: that within 30 seconds of the start of addition of 25 mM KCl a peak where the increase in F/F_{max} was equal to or greater than 0.05 occurred. This identified that the cell in the region of interest was in fact a neurone as the KCl solution results in the depolarisation of cell membranes, and neurones are the only cells present which express voltage-gated calcium channels.

c) **Consistency of responses**: that within 75 seconds of the start of the fourth and fifth capsaicin additions a peak where the increase in F/F_{max} was equal to or greater than 0.075 occurred. This ensured that the selected neurone was responding adequately,

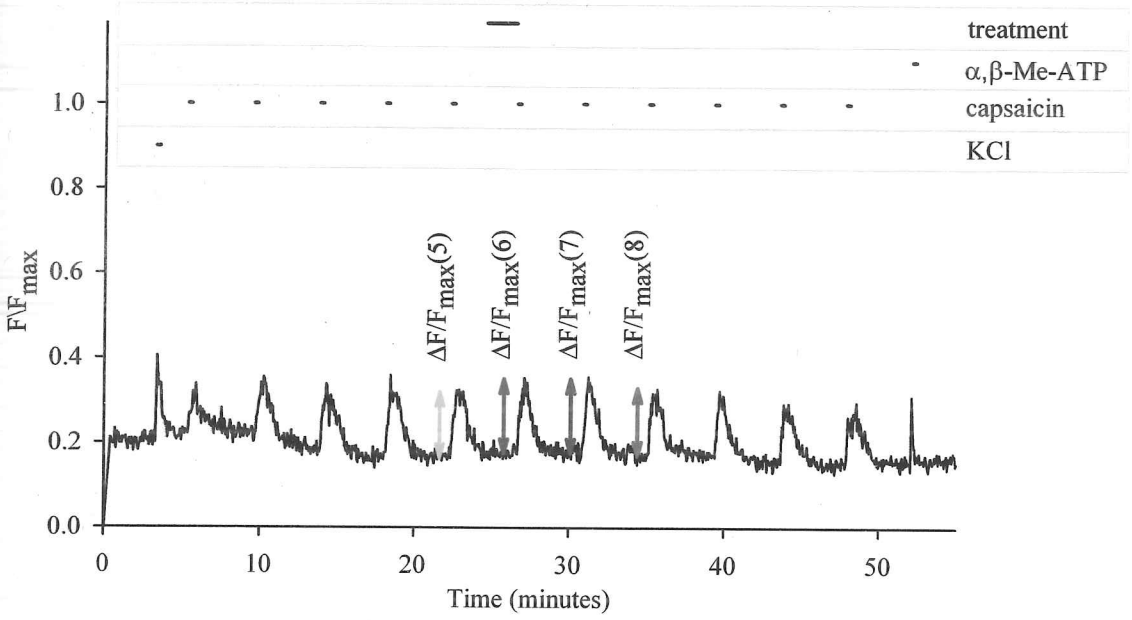

Figure 2.2 Repeated capsaicin additions to a cultured DRG neurone

For the duration of the time points indicated by bars the following solutions are normally added: 25 mM KCl, 100 nM capsaicin and 100 μM α,β-Me-ATP. In addition, the above example trace shows a drug treatment (in this case 100 nM WIN 55,212-2 and 1 μM PGE_2) being applied for two minutes between the fifth and sixth capsaicin additions. The vertical lines with arrowheads adjacent to the calcium transients indicate the increases in intracellular calcium concentration evoked by the fifth, sixth, seventh and eighth capsaicin additions.

and with some consistency, to the addition of capsaicin. Neurones excluded were those that did not respond repeatedly to capsaicin and those that demonstrated dramatic alternation in their response to capsaicin such that alternate peaks were very small. Figure 2.3(a) demonstrates one such excluded neurone.

d) **Artefacts**: that within 30 seconds of the start of addition of HBSS (applied manually) and 0.01 or 0.02% DMSO no peak where the increase in F/F_{max} was equal to or greater than 0.05 occurred. Thus no neurone displaying a response to the physical addition of a test solution (e.g. HBSS) or a response to the vehicle of the test solution (i.e. DMSO solution) was included in the analysis. As such, all responses could be deemed to be due to the active component of the test solution being added.

e) **Adequate recovery from response**: that the baseline prior to the sixth capsaicin addition was within 20% of the value of the baseline prior to the fifth capsaicin addition. This ensured that the neuronal response to the fifth capsaicin addition had adequately subsided before the sixth capsaicin addition. The trace of a neurone excluded by this rule is displayed in Figure 2.3(b).

f) **Stable baseline**: that during the 30 seconds preceding the fifth capsaicin addition all the F/F_{max} readings were within 0.08 in value. This check prevented the inclusion of neurones with a highly erratic baseline, which could confound the automated reading of peak heights. Figure 2.4(a) is a trace of a neurone excluded because of its highly erratic baseline.

g) **Neuronal health**: that the averaged baseline during the 30 seconds prior to the fifth capsaicin addition was no more than 0.125 greater in F/F_{max} value than the averaged baseline during the 30 seconds prior to the addition of 25 mM KCl. This was to exclude those neurones of poor health that demonstrated inadequate recovery from initial treatment with either KCl or capsaicin (Figure 2.4(b)).

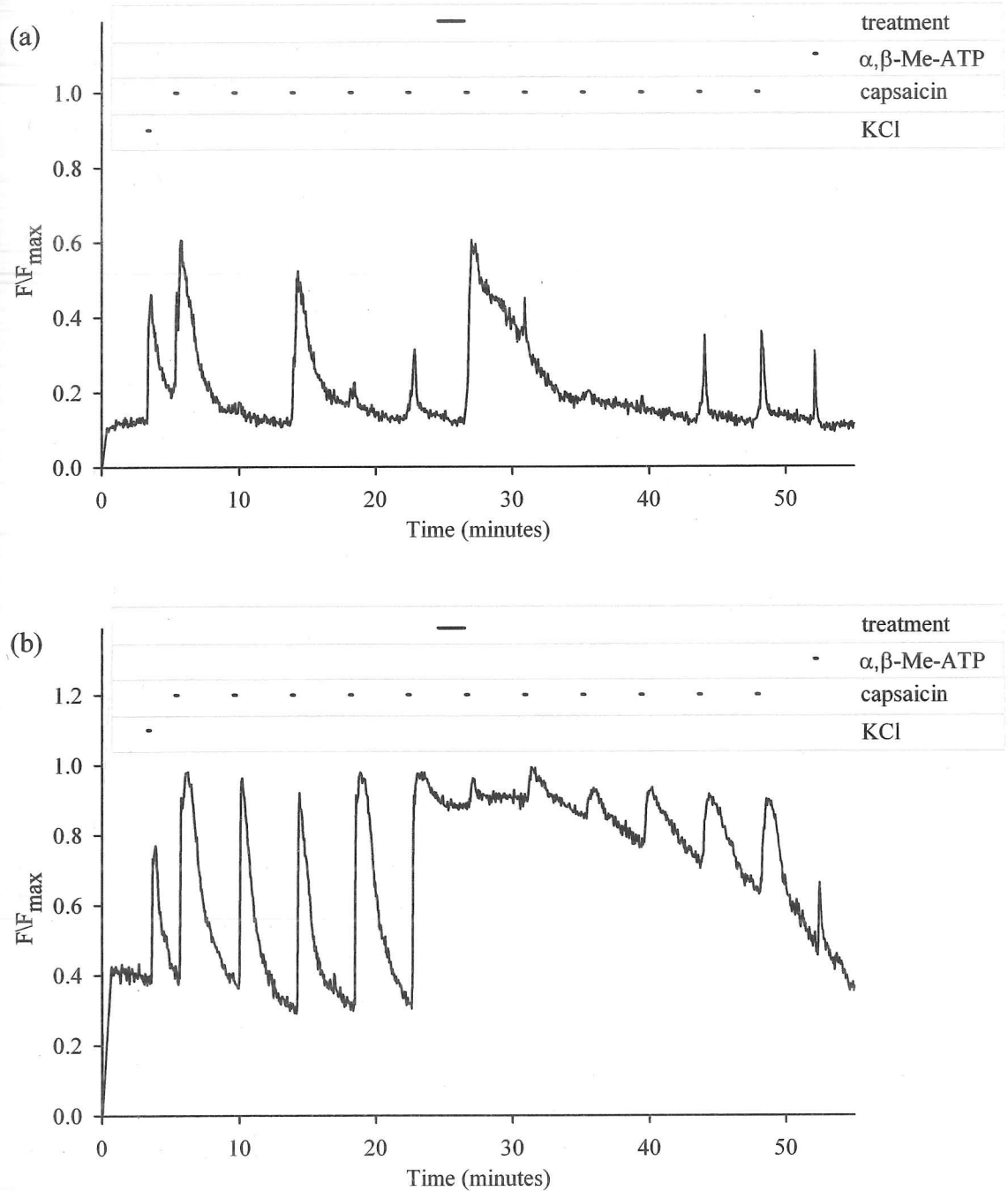

Figure 2.3 Traces exemplifying exclusions made by the automated analysis system.

(a) is a trace which was excluded from analysis because it did not have an adequate response to the fourth addition of capsaicin. (b) is an excluded trace because there was not an adequate recovery in fluorescence after the fifth addition of capsaicin.

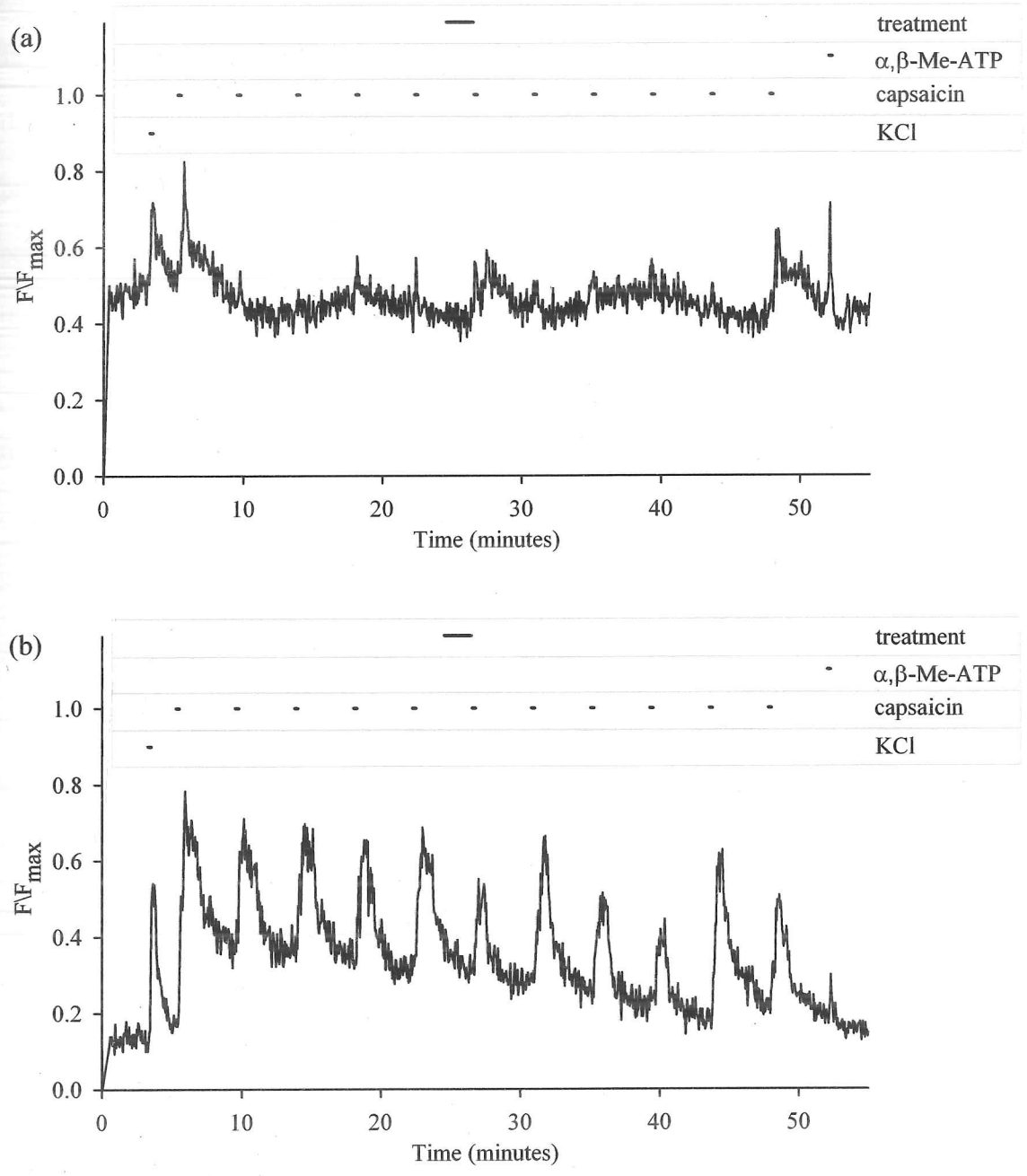

Figure 2.4 Traces exemplifying exclusions made by the automated analysis system. (a) is an excluded trace because of its highly fluctuating baseline. (b) is a trace with a high intracellular calcium concentration that is sustained from the response to the first capsaicin addition. This is indicative of an unhealthy neurone, and as such this trace is excluded from the analysis.

In general, the above limits were set to minimise the risk of including any region of interest demonstrating fluctuations liable to be interpreted as peaks by the automated analysis, while preventing the exclusion of valid regions of interest. They were determined by visually comparing a selection of traces with the manner in which the automated analysis dealt with the corresponding regions of interest.

If all these criteria were satisfied, then the peak values within 75 seconds of the start of the fifth and sixth capsaicin additions were obtained, and then the ratio of the value for the sixth capsaicin addition to the value for the fifth capsaicin addition (Ratio 6/5) was calculated. Similarly, Ratio 7/5, Ratio 8/5, and the ratio of the areas for the peaks evoked by the fifth and sixth capsaicin additions (AreaRatio 6/5) were calculated.

Neurones were determined to be sensitised or not (according to the criteria discussed in Chapter 3), and the percentage of neurones sensitised, as compared with the total number of neurones for which ratios had been calculated, was determined for each coverslip. The mean of the percentages of neurones sensitised for the set of coverslips associated with a particular drug treatment was then calculated, as was the associated standard error of the mean (s.e.m.). The s.e.m. was in fact the estimated standard error of the sample mean, and was calculated by using Microsoft Office Excel 2000 to divide the sample standard deviation of these percentages by the square root of the sample size (i.e. the number of coverslips). The mathematical formula used to calculate the sample standard deviation was as follows:-

$$\sqrt{\frac{\sum_{i=1}^{n}(x_i-\bar{x})^2}{(n-1)}}.$$

The means associated with different drug treatments were then compared with the control data and each other using a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test (SPSS for Windows; version 9.0.1; SPSS Inc.). If the difference between two means had a probability of less than 0.05, it was deemed statistically significant.

In addition to assessing how a drug treatment affected those DRG neurones that repeatedly responded to capsaicin, the phenotype of the general population of neurones present (based upon their responses to capsaicin and α,β-Me-ATP) was examined. In order to characterise as wide a range of DRG neurones as possible, the inclusion criteria listed above were relaxed. Only points (a), (b), (d), and (f) (which ensure no significant dye loss, that the cell is a neurone, that artefacts are absent, and that the baseline is stable) were required to be met in order that responses to capsaicin and α,β-Me-ATP could be assessed. The other inclusion criteria, (c), (e), and (g), were not necessary as consistent, repetitive responses to capsaicin are not required to establish if a neurone responds to capsaicin. This also made it possible to lower the criteria for determining if a neurone responded to capsaicin as there was no need to guard against dramatic alternation in the response to capsaicin. As such, if within 75 seconds of the start of the first, second or fifth capsaicin additions, a peak where the increase in F/F_{max} was equal to or greater than 0.06 occurred, then the neurone was deemed to respond to capsaicin. If within 30 seconds of the start of the addition of α,β-Me-ATP, a peak where the increase in F/F_{max} was equal to or greater than 0.06 occurred, then the neurone was deemed to respond to this agonist. Using this same criterion (with respect to inclusion, and the limits for peaks evoked by capsaicin), neurones that were non-responsive to the first, second, third, fourth and fifth capsaicin additions but did respond to the sixth capsaicin addition (after exposure to the drug treatment) were identified, as they were believed to be correlates of the 'silent' nociceptors reported elsewhere (Schmidt *et al.*, 1995).

The phenotype of the neurones repeatedly responding to capsaicin, when the neurones were exposed to different treatments, was compared using the Chi-square test, assuming that there was no difference in the proportion of the different phenotypes. For further information on the Chi-square test see Brown and Rothery (1994).

2.3 Immunocytochemistry

Visualisation of epitopes

Coverslips of neurones were washed three times with DPBS-1 before being exposed to 1μM phorbol 12-myristate 13-acetate (PMA; Alexis), 5μM BAM (8-22) (courtesy of AstraZeneca) or DPBS-1 for a specified time. The neurones were then quickly fixed for 15 minutes at room temperature using 4% paraformaldehyde (w/v) with 4% sucrose (w/v) dissolved in Dulbecco's Phosphate Buffered Saline (without calcium and magnesium; DPBS-2; Invitrogen; Cat No. 14190). For the rest of the protocol all solutions contained 1% (v/v) fish skin gelatin to prevent non-specific binding, and prior to each step the coverslips were washed three times for five minutes with DPBS-2.

Firstly, the membranes of the neurones were permeabilised by exposing them to DPBS-2 containing 0.2% (v/v) Triton X-100 (Sigma) for 30 minutes at room temperature. Next, the neurones were exposed for 1 hour at room temperature to a specified dilution of primary antibody (see Table 2.1) in DPBS-2 containing 0.05% (v/v) Triton X-100 and 0.5% (v/v) sodium azide (antibody solution) before being left with the primary antibody solution overnight at 4°C. The neurones were then exposed for 1 hour at room temperature to a donkey anti-rabbit IgG antibody conjugated to the Alexa Fluor 488 dye (Molecular Probes) diluted 1 in 200 in antibody solution. Coverslips were then mounted onto slides using Immu-Mount (Thermo Shandon).

Antibody target	Antibody type	Source of antibody	Dilution used
PKC-δ	Rabbit (polyclonal)	Non-commercial (Olivier and Parker, 1991)	1 in 1000
PKC-ε	Rabbit (polyclonal)	Non-commercial (V.Vellani after Schaap *et al.* (1989))	1 in 1000
PKC-ζ	Rabbit (polyclonal)	Upstate biotechnology	1 in 500

Table 2.1 Primary antibodies and dilutions used in immunocytochemistry studies

Analysis

Coverslips were examined using a Nikon Eclipse TE300 microscope, with a 60x, oil-immersion Plan Apo objective, connected to a Bio-Rad micro-Radiance Confocal Scanning system (model MRAG2) to detect the emission from the Alexa Fluor 488 dye, which was excited by an air cooled argon/ion laser filtered to produce a beam of 488 nm. The emission from the Alexa Fluor 488 dye was isolated using a 560 nm dichroic mirror, and a HQ 515/30 filter. The photomultiplier gain and iris settings, and the zoom setting were optimised for each region of the coverslip examined so as to provide the best conditions for the visualisation of PKC translocation. Fifteen random images were collected from each coverslip using the Slow scan speed and a Kalman filter (n=3). These images were later analysed using Confocal Assistant (version 4.0; Todd Clark Brelje) and the neurones present in them were scored positive (for example as Figure 4.13(c)) or negative (for example as Figure 4.13(a)) for clear, visible translocation of the PKC isoform being examined. The percentage of neurones showing translocation was then calculated for each coverslip. For each isoform, the mean

percentage (and s.e.m.) of those neurones showing translocation was calculated for each of the treatments. These percentages were then compared using a one-way ANOVA with a Bonferroni post hoc test (SPSS for Windows; version 9.0.1; SPSS Inc.) to assess whether any of the treatments caused a significant increase in translocation.

2.4 Electrophysiology

Whole cell patch clamping

Petri dishes of neurones that had been cultured for 1 to 3 days were washed twice with DPBS-1 before being filled with HBSS adjusted to 325(±3) mOsmoles with sucrose. A neurone of interest—typically one with a healthy membrane appearance, an ellipsoid cell body, and off-centre nucleus—was then identified using a Zeiss IM35 microscope with a 10x objective. With the neurone centred in the field of view, perfusion tubes connected to the rapid solution changer (RSC-100; Bio-Logic) were lowered into the petri dish such that they were just clear of the bottom, to allow movement without disturbing the petri dish, and at the edge of the field of view.

Pipettes of resistance 2-6 MΩ were pulled from borosilicate glass capillaries (GC120F-10; Harvard) using a Flaming Brown micropipette puller (model P-80/PC; Sutter Instrument Co.). These were filled with intracellular solution (in mM: 135 KCl; 1.6 $MgCl_2$; 10 HEPES; 2 EGTA; 2.5 Mg-ATP; 0.2 Li-GTP; adjusted to pH 7.3 with KOH, and 310-311 mOsmoles with sucrose) before being inserted into a pipette holder containing a AgCl wire. Once the pipette holder was connected to the headstage of the patch clamp amplifier (L/M-PC; List-Medical), the pipette was lowered into the HBSS in the petri dish thus completing the circuit

with the AgCl reference electrode in the dish. The pipette offset on the amplifier was then adjusted to correct for the potential difference between the pipette and bath solutions. Next, a gigaohm seal was achieved between the pipette and the plasma membrane of the neurone. The pipette capacitance was compensated by the amplifier circuitry, and the holding membrane potential set to –80 mV. Suction was then applied to the pipette, and the whole cell patch-clamp configuration was achieved. Using the amplifier circuitry, the membrane capacitance was compensated, and the series resistance (with values generally in the range of 7.4 to 44.0 MΩ) was generally compensated by between 41% and 51%.

Time course

Once a successful whole cell patch-clamp recording had been achieved, the time course protocol was started. The neurone was superfused with HBSS, and every 30s, for 1s, was exposed to 100nM capsaicin. While these exposures were repeated, 5μM BAM (8-22) was perfused after 150s for 5 minutes. After 25 exposures to capsaicin, 10μM α,β-Me-ATP was applied for 5s in order to test for the presence of $P2X_3$ receptors (Ueno *et al.*, 1999).

The whole-cell current evoked by capsaicin was sampled at 5kHz and filtered at 1kHz through a 6-pole Bessel filter. All experiments were carried out at room temperature (21±1°C), and the length of the longest axis of the neurone cell body was measured using an eyepiece graticule.

Analysis

Recordings were analysed using Clampfit (version 8.2; Axon Instruments Inc.). A 5Hz Gaussian lowpass filter was applied to the data, and the baseline was corrected to zero, before measuring the peak current, and τ for a one-term, standard exponential fitted to the decay of the response:-

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau i} + C,$$

using the Simplex fitting method (with zero-shift) (for further information on this fitting method see the Clampfit instruction manual).

Some neurones gave an initial response to capsaicin but the subsequent response was small (less than 25 pA) or absent; such neurones were excluded. Neurones were also scored for the presence of $P2X_3$ receptors based upon a response to α,β-Me-ATP. The method of determining if a neurone was sensitised by BAM (8-22) is detailed in Chapter 5.

Chapter 3

Results: Cannabinoids

Cannabinoid receptors are the cellular site at which the active ingredients of cannabis mediate their effects (Devane *et al.*, 1988). Two such receptors have been clearly identified so far: CB_1 (Matsuda *et al.*, 1990) and CB_2 (Munro *et al.*, 1993). The analgesic action of cannabinoids (Abel, 1979) has been found to be mediated via these receptors. This action was originally found to occur via a mechanism within the central nervous system (CNS) (Meng *et al.*, 1998). Recent reports, however, have shown the presence of cannabinoid receptors on primary afferent neurones (Hohmann and Herkenham, 1999b; Ahluwalia *et al.*, 2000) and that cannabinoids can modulate nociception by a peripheral mechanism of action (Calignano *et al.*, 1998; Richardson *et al.*, 1998b). In addition, functional cannabinoid receptors have been shown on cultured dorsal root ganglion (DRG) neurones (Ross *et al.*, 2001; Tognetto *et al.*, 2001; Millns *et al.*, 2001). A pharmaceutical based upon activating peripheral cannabinoid receptors, but not central cannabinoid receptors, would be clinically beneficial as it would avoid psychoactive side effects which make cannabis less desirable as an analgesic drug.

It was therefore decided to examine at the cellular level how the activation of cannabinoid receptors affected the response of nociceptive neurones. This can be done in a controlled *in vitro* setting as cultured DRG neurones are known to express the characteristics of nociceptors *in vivo* (Baccaglini and Hogan, 1983; Gold *et al.*, 1996a; Cesare and McNaughton, 1996). Of those primary afferents responding to noxious stimuli, a subset are identified by the expression of the transient receptor potential vanilloid 1 (TRPV1) cation channel (Tominaga *et al.*, 1998), and capsaicin was used to activate these receptors (Caterina *et al.*, 1997).

Capsaicin sensory neurones are known to show tachyphylaxis on repeated stimulation (Docherty *et al.*, 1996; Koplas *et al.*, 1997), and thus in order to be able to easily assess the effect that a drug treatment has, it was necessary to repeatedly apply capsaicin. Repeated

application ensured that any tachyphylaxis in the response to capsaicin had plateaued before the effect of the drug was assessed. Neurones were loaded with a fluorescent marker for calcium (Fluo-4) to monitor their intracellular calcium level as a means of measuring the increase in intracellular calcium concentration from TRPV1 activation. After five additions of 100nM capsaicin, the drug treatment was applied. Six further capsaicin additions were made, before an application of 100μM α,β-Me-ATP was made in order to test for the expression of ATP-gated ion channels containing the $P2X_3$ subunit (Figure 2.2). This allowed the phenotype of the nociceptive neurones monitored to be further investigated.

For neurones responding repeatedly to capsaicin, the ratio of the calcium transients evoked by the fifth and sixth capsaicin additions was calculated (Ratio 6/5). In order to assess if a treatment resulted in a sensitisation of the response of neurones to capsaicin, control data in the absence of drug treatment was collected (Figure 3.1(a); black bars; n=90). These data were seen to follow a normal distribution with a mean of slightly less than one (0.917308; Figure 3.1(a); green dashed line) indicating that although the tachyphylaxis had subsided by the fifth capsaicin addition, it was still ongoing to some extent. The 95% two-tailed confidence limits for Ratio 6/5 were then determined for the control data by utilising the range around the mean of 1.96 times the sample standard deviation (0.076276 and 1.75834; Figure 3.1(a); red and blue dashed line, respectively). This enabled data from a drug treatment experiment to be compared with it. 1.92±1.92% of neurones from the control data fell above the upper confidence limit which is close to the expected 2.5% given the 95% confidence limit.

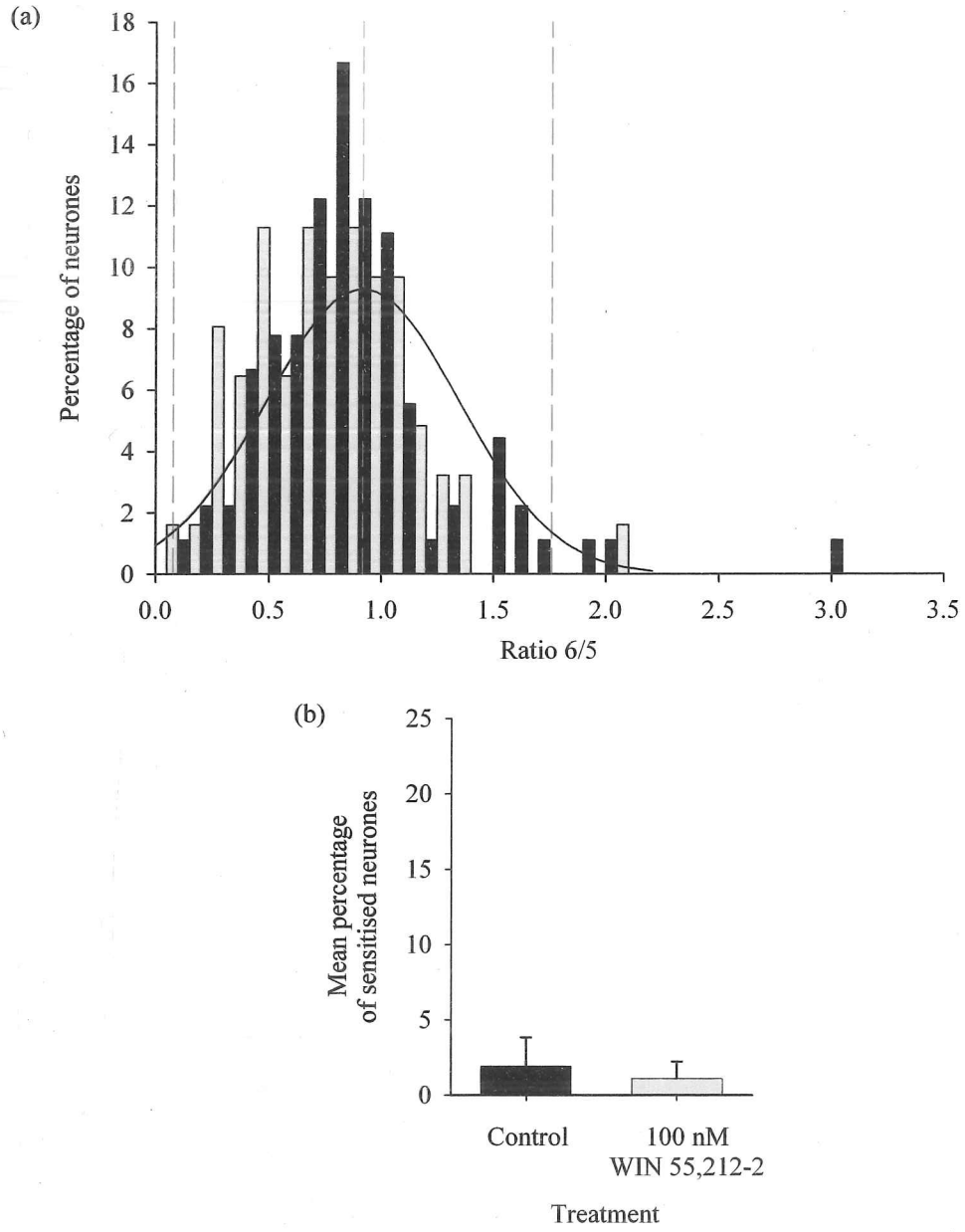

Figure 3.1 WIN 55,212-2 does not affect nociceptor response to 100 nM capsaicin.

(a) A histogram displaying the percentage of neurones from control (black, *n*=90) and treatment (grey, *n*=62) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth transient (Ratio 6/5). The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (n_{exp}=13) and treatment (n_{exp}=10) experiments where the value of Ratio 6/5 is greater than the upper 95% confidence limit in figure (a).

Cannabinoid receptor activation in a non-sensitised model

In order to test the effect of cannabinoid receptor activation on the response of these TRPV1 expressing neurones, it was necessary to choose an appropriate agonist. The cannabinoid receptor agonist WIN 55,212-2 was selected because of its known efficacy at both CB_1 and CB_2 receptors (Pertwee, 2000) thus ensuring that both known cannabinoid receptors were activated. 100 nM WIN 55,212-2 was applied for two minutes prior to the sixth capsaicin addition. The Ratio 6/5 data for neurones treated with WIN 55,212-2 (Figure 3.1(a); grey bars; n=62) was compared against the confidence limits determined for the control data. If a neurone had a ratio that fell below the lower 95% confidence limit, it was deemed to have been desensitised, and if it had a ratio that was above the upper 95% confidence limit, it was deemed to have been sensitised. The mean percentages (and the standard errors of the means) of neurones sensitised (Control=1.92±1.92%; 100 nM WIN 55,212-2=1.11±1.11%; Figure 3.1(b)) were obtained for each set of coverslips (Control n_{exp}=13; WIN 55,212-2 n_{exp}=10), and no significant difference between the control and WIN 55,212-2 data was seen. Because of the magnitude of the variance of the control data, the range within which neurones could be deemed to be desensitised (i.e. from 0 to the lower confidence limit) was rather small and prevented meaningful analysis (e.g. no control neurones fell within this range and for the WIN 55,212-2 experiments only 1±1% were present). If the neurones were less variable in their responses, desensitisation might have been easier to quantify. This of course raises the problem of being able to assess if cannabinoid receptor activation results in attenuation of the response of nociceptive neurones in a non-sensitised state.

Prostaglandin E_2 (PGE_2) as a model of sensitisation

In vivo studies showed that the activation of cannabinoid receptors in the periphery is antihyperalgesic (Calignano *et al.*, 1998; Richardson *et al.*, 1998b). Therefore, given that the sensitisation of nociceptive neurones underlies primary hyperalgesia (Meyer & Campbell 1981; La Motte *et al.*, 1982), it was decided to examine the effect of WIN 55,212-2 when the cultured neurones were in a sensitised state. In order to obtain sensitised neurones it was necessary to find a suitable agent. PGE_2 is a major component of inflammatory exudates, and plays a role in the initiation of primary hyperalgesia. Previous reports have shown that it is able to sensitise nociceptive neurones in culture (Baccaglini and Hogan, 1983; Nicol and Cui, 1994; Cui and Nicol, 1995; Stucky *et al.*, 1996; Gold *et al.*, 1996a) via the activation of the cyclic 3',5'-adenosine monophosphate/Protein Kinase A (cAMP/PKA) pathway (Taiwo *et al.*, 1989; Pitchford and Levine, 1991; Taiwo and Levine, 1991; Cui and Nicol, 1995). This is especially relevant given that cannabinoid receptors are known to be coupled to the inhibition of adenylate cyclase which generates cAMP (Howlett *et al.*, 1986) and thus could be modulating the primary hyperalgesia via a mechanism involving the inhibition of cAMP production.

1 μM PGE_2 was applied for two minutes prior to the sixth capsaicin addition and resulted in an increase in the calcium transient resultant from the sixth application of capsaicin (Figure 3.2). The Ratio 6/5 data for neurones treated with PGE_2 (Figure 3.3(a); grey bars; n=218) was compared against the confidence limits determined for the control data. No desensitisation was seen in the PGE_2 experiments. In control experiments (n_{exp}=13), 1.92±1.92% neurones fell above the upper confidence limit, compared with a significantly higher (at the 0.05 level) 15.64±4.14% in the PGE_2 experiments (n_{exp}=16; Figure 3.3(b)).

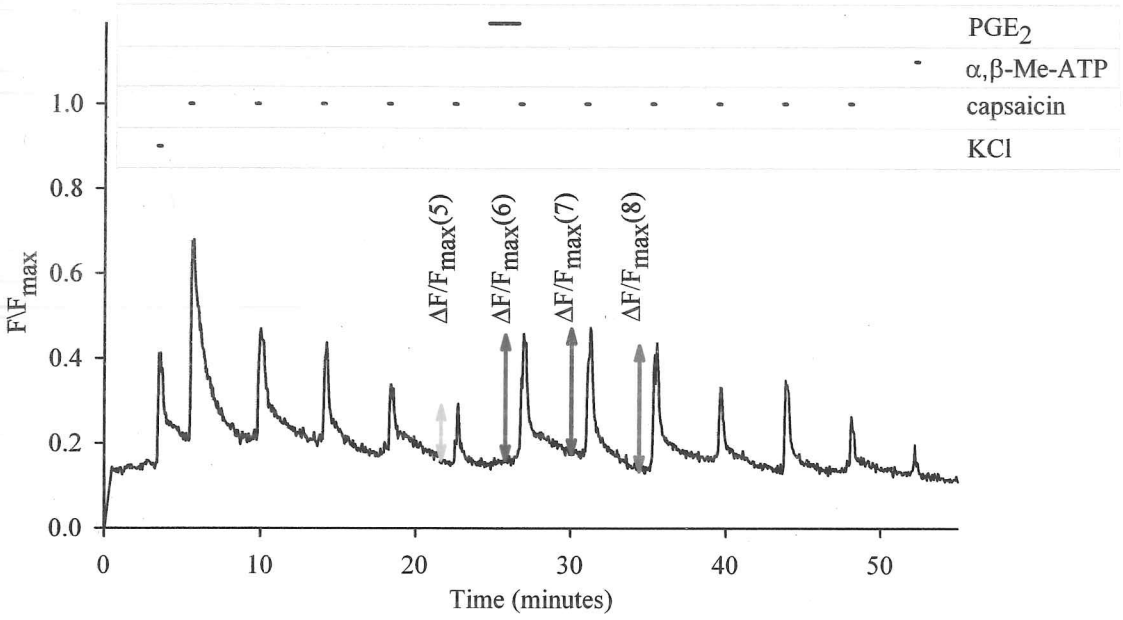

Figure 3.2 PGE_2 sensitises a cultured neonatal rat DRG neurone to capsaicin.

A plot of the intracellular calcium concentration of the neurone (as measured by F/F_{max}) over time. For the duration of the time points indicated by bars the following solutions were added: 25 mM KCl, 100 nM capsaicin, 1 μM PGE_2 and 100 μM α,β-Me-ATP. The vertical lines with arrowheads adjacent to the calcium transients indicate the increases in intracellular calcium concentration evoked by the fifth, sixth, seventh and eighth capsaicin additions.

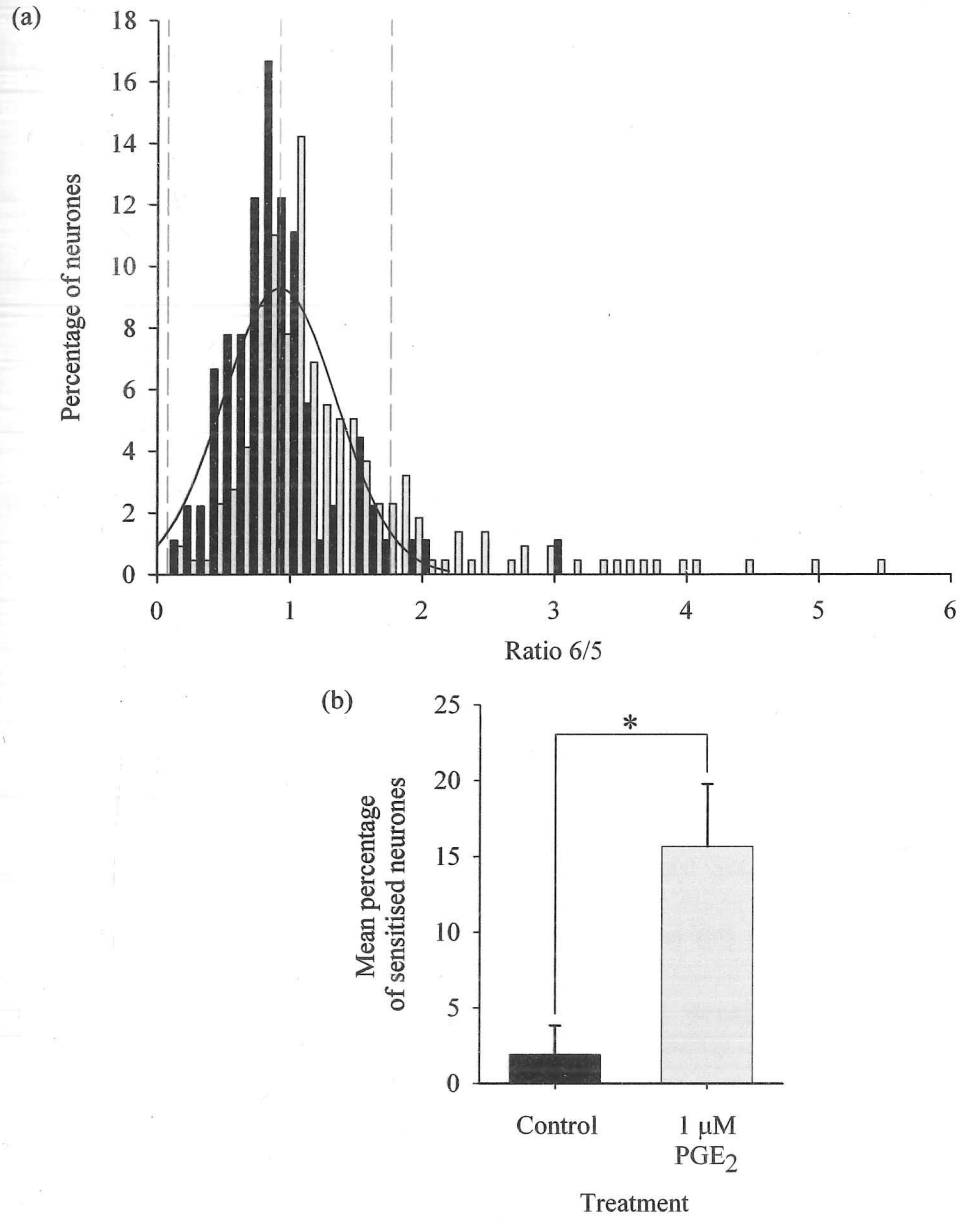

Figure 3.3 PGE_2 sensitises nociceptor response to 100 nM capsaicin.

(a) A histogram displaying the percentage of neurones from control (black, *n*=90) and treatment (grey, *n*=218) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth transient (Ratio 6/5). The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (n_{exp}=13) and treatment (n_{exp}=16) experiments where the value of Ratio 6/5 is greater than the upper 95% confidence limit in figure (a). * indicates that the mean difference is significant at the 0.05 level.

In addition to looking simply at the transient height, the ratio of the area under the transients evoked by the fifth and sixth capsaicin additions was calculated (AreaRatio 6/5) (Figure 3.4(a)). An increased area, with a broadened response, after treatment with PGE_2 could be indicative of a decreased off rate between capsaicin and TRPV1 resulting in prolonged activation. Such an effect is evident in Figure 3.2. The mean for control data in this case was also just less than one (0.989988), but the sample standard deviation was larger resulting in an upper 95% confidence limit of 2.62513. Again, when the percentage of neurones sensitised was examined (Figure 3.4(b)), there was a significant difference (at the 0.05 level) between the control data (4.01±2.84%) and the PGE_2 data (25.82±5.97%). Thus the application of 1 μM PGE_2 for two minutes results in a significant proportion of neurones being sensitised in their response to capsaicin, and a direct effect on TRPV1 is suggested. The fact that a greater percentage of neurones (approximately 26%) were seen to be sensitised by PGE_2 when AreaRatio 6/5 was examined compared with Ratio 6/5 (where only approximately 16% of neurones were sensitised) implies that a direct sensitisation of TRPV1 plays a more significant role than a sensitisation of other components that may be involved in the generation of the calcium transient. The involvement of other components in the generation of the calcium transient, such as voltage-gated calcium channels, is discussed in Chapter 6 (Section 3).

The duration of this sensitisation was assessed by examining the ratios of the calcium transients evoked by the seventh and eighth capsaicin additions to the fifth capsaicin addition (Figure 3.5). There was no significant difference between these ratios for control and PGE_2 experiments. The reason for the high mean percentage of the control Ratio 7/5 is because of a single coverslip giving an outlier result. Removing it from the analysis, however, did not result in any change to the significance. Thus the sensitisation is limited to the capsaicin addition immediately after the PGE_2 addition.

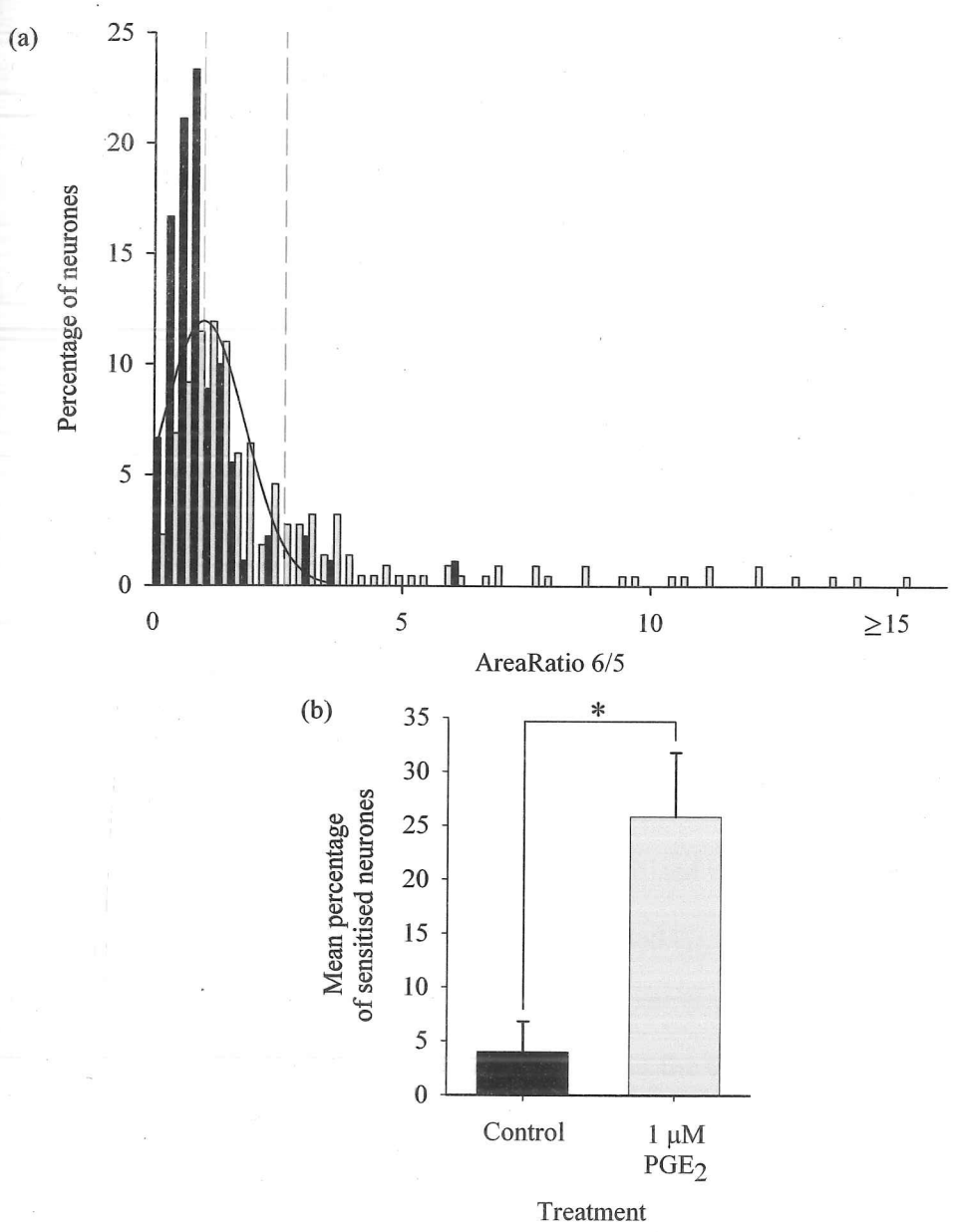

Figure 3.4 PGE_2 increases the area of the capsaicin-evoked calcium transient.

(Same as figure 3.3 except that AreaRatio 6/5 is being examined)

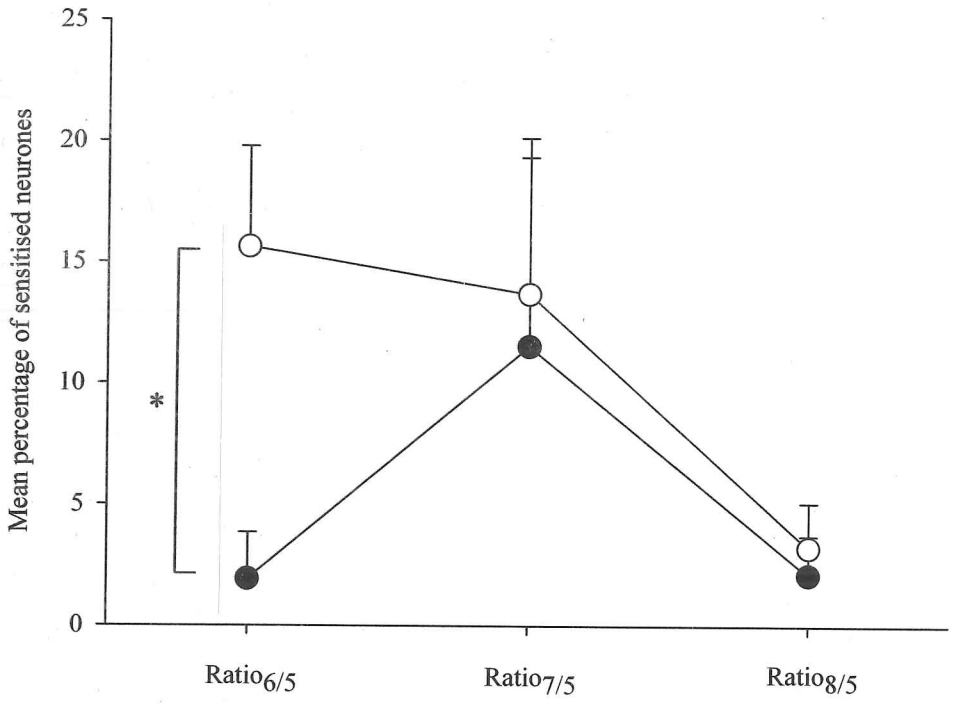

Figure 3.5 Sensitisation by PGE_2 is restricted to the subsequent capsaicin addition.

Plot of the mean percentage (with s.e.m.) of sensitised neurones identified by $Ratio_{6/5}$, $Ratio_{7/5}$, and $Ratio_{8/5}$ in control experiments (filled circles; n_{exp}=13, 13, 12) and experiments with a two minute treatment with 1 μM PGE_2 (open circles; n_{exp}=16,16,16).

* indicates that the mean difference is significant at the 0.05 level.

The phenotype of the neurones present in these experiments was assessed in order to see if the subset of neurones sensitised by PGE_2 differed from either the general population of neurones or the subset of neurones which repeatedly responded to capsaicin. Neurones fell into four categories: those responsive to neither capsaicin nor α,β-Me-ATP (Figure 3.6(a)), those responsive to capsaicin only (Figure 3.6(b)), those responsive to both capsaicin and α,β-Me-ATP (Figure 3.7(a)), and those responsive to α,β-Me-ATP only (Figure 3.7(b)). Figure 3.8(a) displays the number of neurones falling into each category for all of the PGE_2 experiments, and Figure 3.8(b) provides a comparison amongst all neurones (black), those that met the criteria laid out in Chapter 2 for a stable and noise-free repeated capsaicin response (light grey), and those out of this latter category that were sensitised by PGE_2 (dark grey). From Figure 3.8(b) it appears that neurones repeatedly responding to capsaicin are just as likely to respond to α,β-Me-ATP as to be non-responsive to it. However, those responding to α,β-Me-ATP appear to be less likely to be sensitised compared to those that do not respond though this is not statistically significant (Chi-square test).

Figure 3.9 shows a recording from a cultured DRG neurone that is a correlate of the 'silent' nociceptors described in the introduction (Schmidt *et al.*, 1995) which become responsive in the presence of a sensitising factor. In experiments where PGE_2 is applied for two minutes 9.59% of neurones were deemed to be 'silent' nociceptors. By comparison, only 0.81% of neurones in control experiments met the criteria for 'silent nociceptors'.

Cannabinoid receptor activation in a sensitised model

Using the above model of sensitisation, 100 nM WIN 55,212-2 was co-applied with 1 μM PGE_2 in order to activate cannabinoid receptors while nociceptive neurones were in a

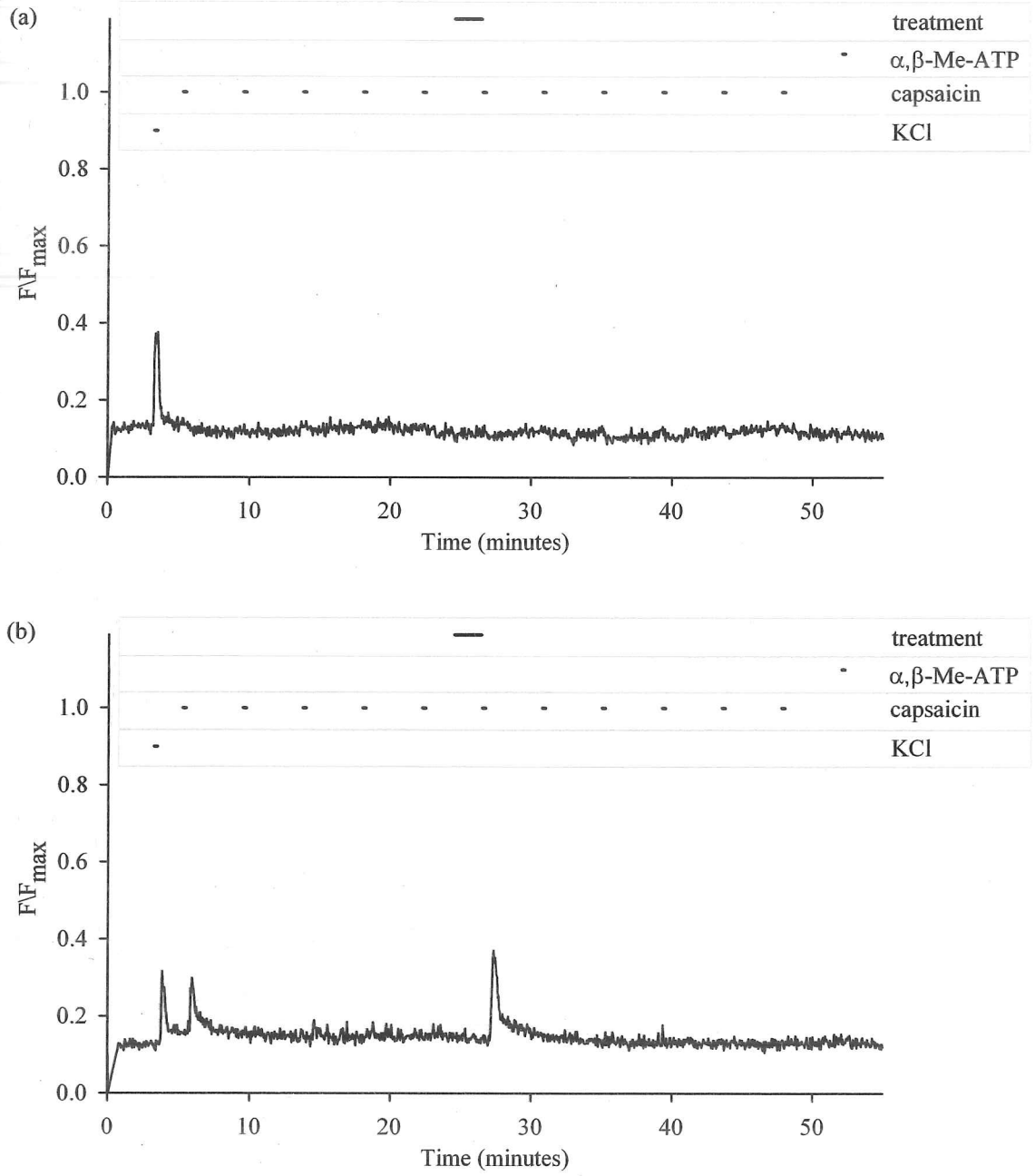

Figure 3.6 Example plots of DRG neurones that Ratio 6/5 values were not calculated for. (a) shows a neurone that is not responsive to capsaicin or α,β-Me-ATP. (b) shows a neurone responsive to capsaicin only. The plots are of the intracellular calcium concentration of the neurones (as measured by F/F_{max}) over time. For the duration of the time points indicated by bars the following solutions were added: 25 mM KCl, 100 nM capsaicin and 100 μM α,β-Me-ATP.

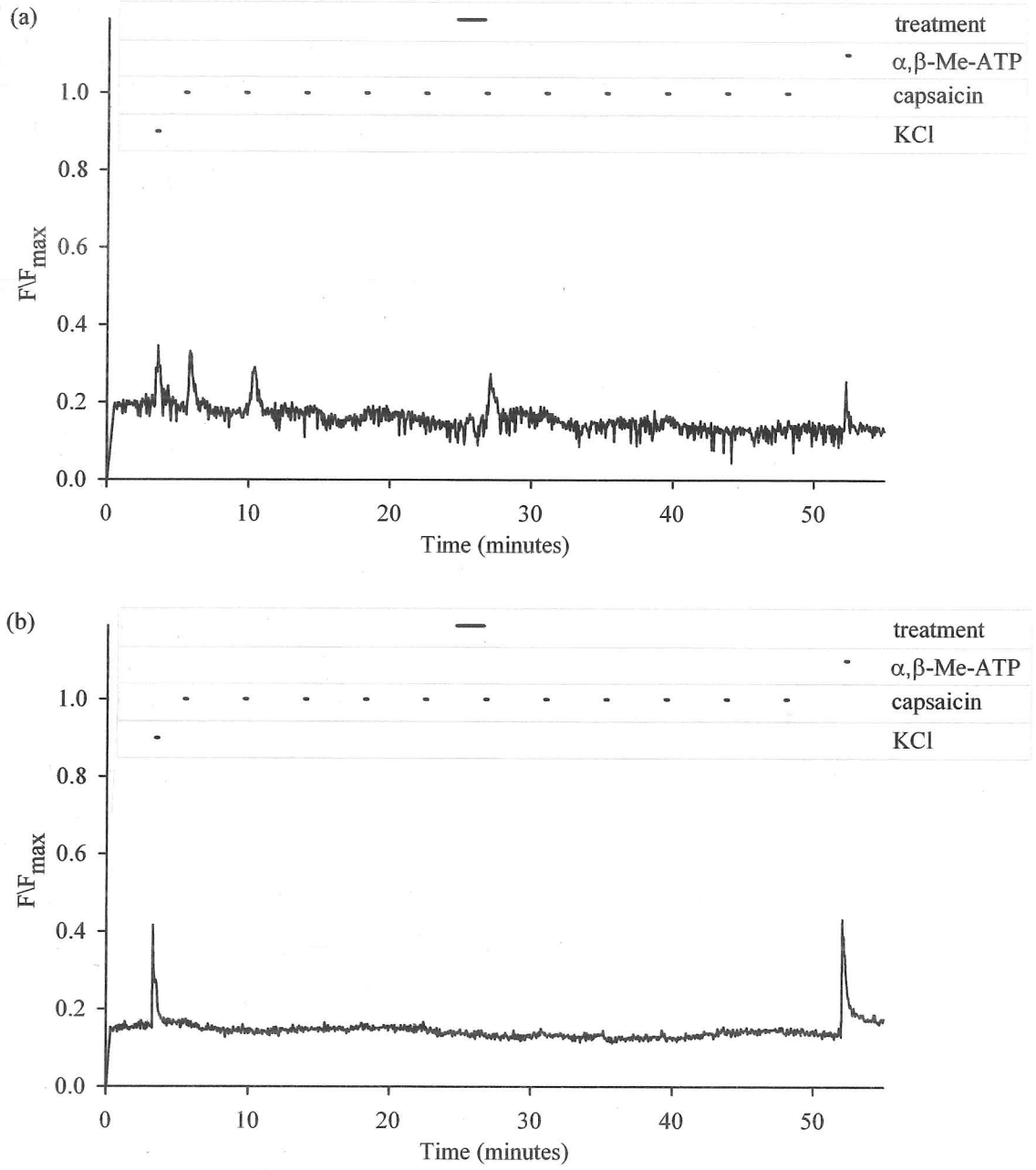

Figure 3.7 Example plots of DRG neurones that Ratio 6/5 values were not calculated for. (a) shows a neurone that is responsive to both capsaicin and α,β-Me-ATP. (b) shows a neurone responsive to α,β-Me-ATP only. The plots are of the intracellular calcium concentration of the neurones (as measured by F/F_{max}) over time. For the duration of the time points indicated by bars the following solutions were added: 25 mM KCl, 100 nM capsaicin and 100 μM α,β-Me-ATP.

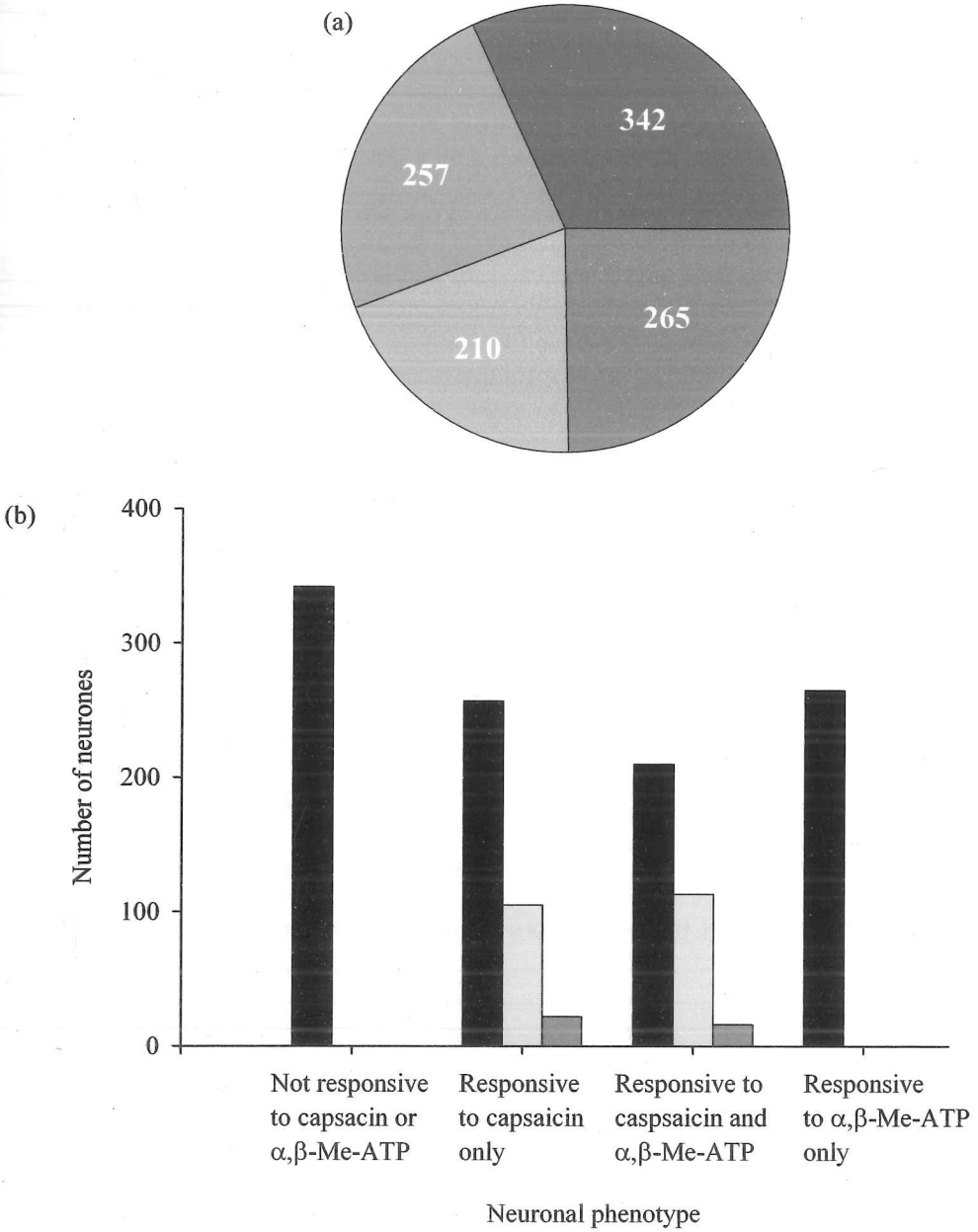

Figure 3.8 Characterisation of DRG neurones from PGE_2 sensitisation experiments. (a) A pie chart displaying the number of neurones analysed that responded to neither 100 nM capsaicin nor 100 μM α,β-Me-ATP (red), to only capsaicin (orange), to both capsaicin and α,β-Me-ATP (yellow), and to only α,β-Me-ATP (green). (b) A bar chart displaying the same information as in (a) (black), and in addition, displaying the number of neurones in each category for the subset of neurones that gave a stable response to capsaicin (light grey), and for the neurones of this subset that had a Ratio 6/5 value greater than the upper 95% confidence limit denoted in figure 3.3(a) (dark grey).

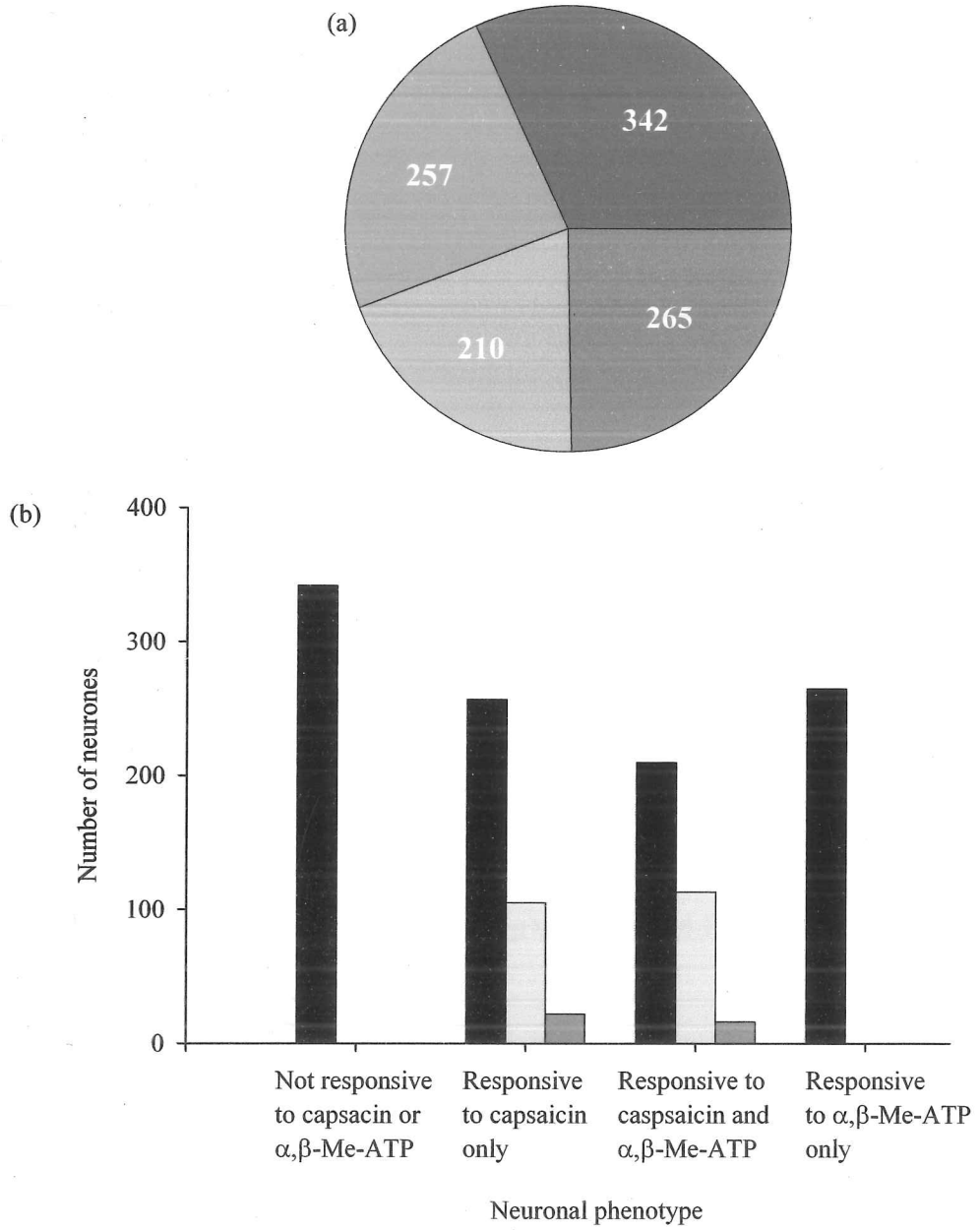

Figure 3.8 Characterisation of DRG neurones from PGE_2 sensitisation experiments. (a) A pie chart displaying the number of neurones analysed that responded to neither 100 nM capsaicin nor 100 μM α,β-Me-ATP (red), to only capsaicin (orange), to both capsaicin and α,β-Me-ATP (yellow), and to only α,β-Me-ATP (green). (b) A bar chart displaying the same information as in (a) (black), and in addition, displaying the number of neurones in each category for the subset of neurones that gave a stable response to capsaicin (light grey), and for the neurones of this subset that had a Ratio 6/5 value greater than the upper 95% confidence limit denoted in figure 3.3(a) (dark grey).

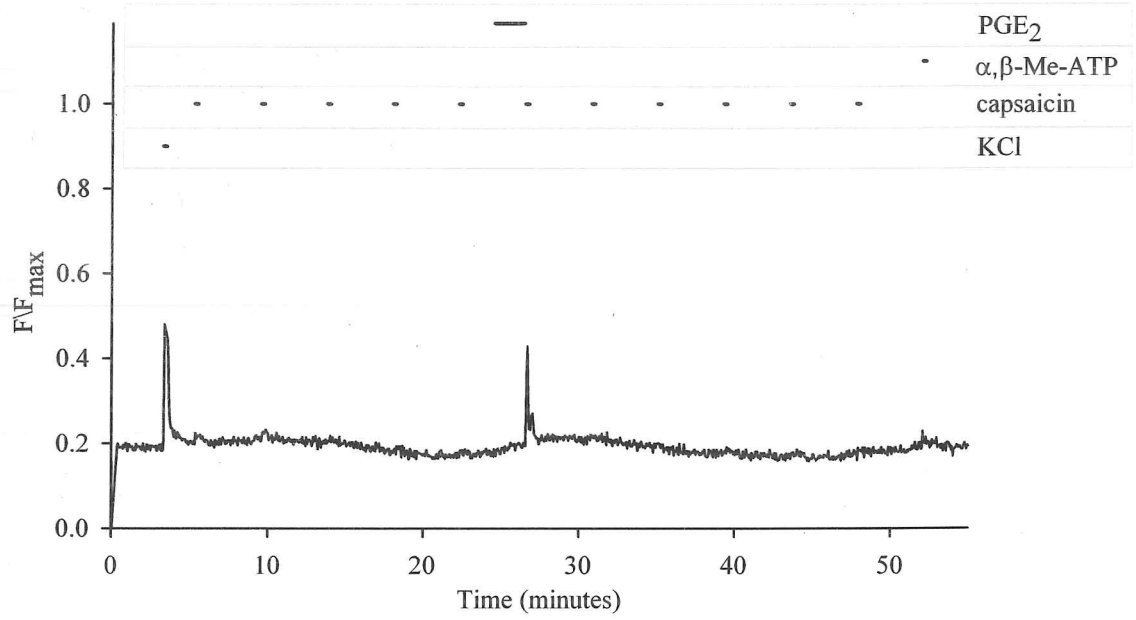

Figure 3.9 Example plot of a 'silent' nociceptor.

The plot is of the intracellular calcium concentration of the neurone (as measured by F/F_{max}) over time. For the duration of the time points indicated by bars the following solutions were added: 25 mM KCl, 100 nM capsaicin, 1 μM PGE_2 and 100 μM α,β-Me-ATP.

sensitised state. The Ratio 6/5 data for neurones treated with PGE_2 and WIN 55,212-2 (Figure 3.10(a); grey bars; n=160) was compared against the confidence limits determined for the control data. Neither in the control nor the PGE_2 and WIN 55,212-2 experiments was any desensitisation seen. In control experiments (n_{exp}=13), 1.92±1.92% neurones fell above the 95% confidence limit, compared with 10.03±3.47% in the PGE_2 and WIN 55,212-2 experiments (n_{exp}=16; Figure 3.10(b)). These two values were not significantly different however, and neither was the percentage of neurones sensitised in the presence of WIN 55,212-2 and PGE_2 from the percentage sensitised in the presence of PGE_2 only (15.64±4.14%).

Again the ratio of the area under the transients evoked by the fifth and sixth capsaicin additions was calculated (AreaRatio 6/5) (Figure 3.11(a)). As with the Ratio 6/5 data, there was no significant difference between the control data (4.37±2.41%), and the PGE_2 and WIN 55,212-2 data (16.78±5.04%) (Figure 3.11(b)).

As with the PGE_2 experiments, the phenotype of the neurones present in these experiments was assessed. Figure 3.12(a) displays the number of neurones falling into each category. Figure 3.12(b) provides a comparison amongst all neurones (black), those that met the criteria laid out in Chapter 2 for a stable and noise-free repeated capsaicin response (light grey), and those sensitised by PGE_2 (dark grey). Once again, those neurones not responding to α,β-Me-ATP appear to be preferentially sensitised, and in this case this is statistically significant (Chi-square test, significant at 0.05 level, χ^2=5.538).

In these experiments where PGE_2 and WIN 55,212-2 were applied for two minutes, 5.53% neurones were deemed to be 'silent' nociceptors. These 'silent' nociceptors are dealt with separately from those neurones which are included in the Ratio 6/5 analysis because their lack of a noticeable response to previous capsaicin additions makes it mathematically impossible for a sensible ratio to be calculated. It also seems inappropriate to simply include them as

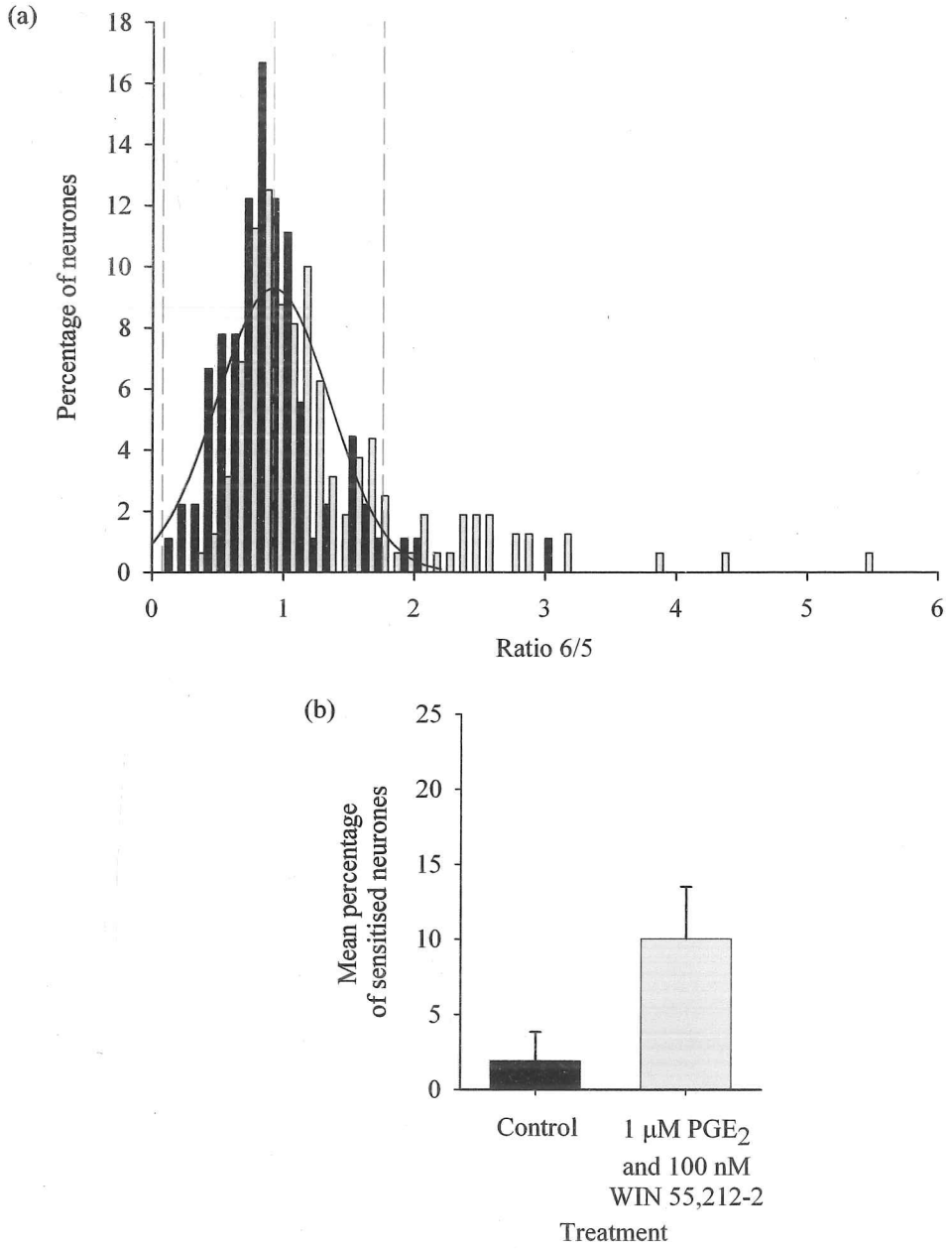

Figure 3.10 WIN 55,212-2 does not significantly inhibit sensitisation by PGE_2.

(a) A histogram displaying the percentage of neurones from control (black, n=90) and treatment (grey, n=160) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth transient (Ratio 6/5).

The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (n_{exp}=13) and treatment (n_{exp}=16) experiments where the value of Ratio 6/5 is greater than the upper 95% confidence limit in figure (a).

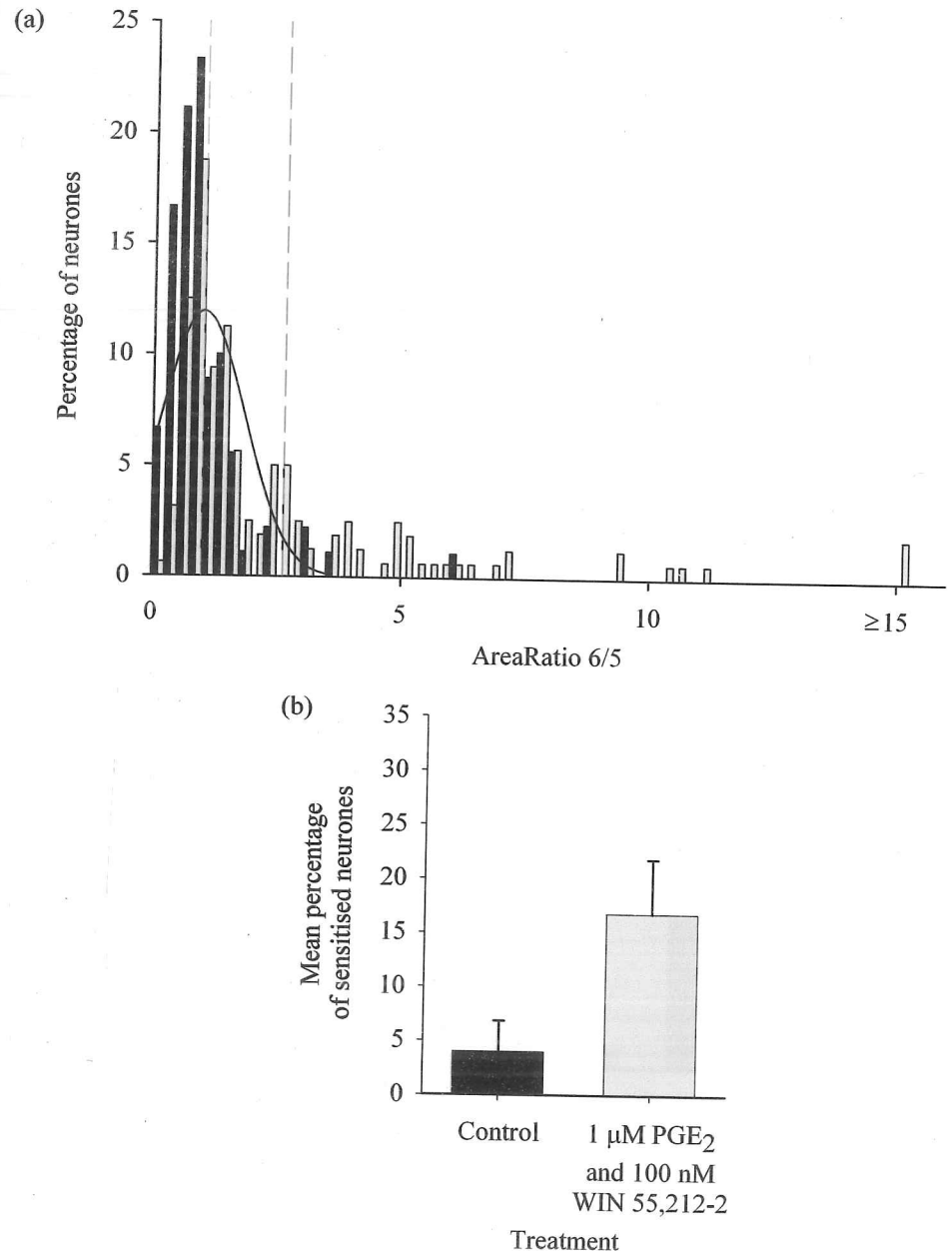

Figure 3.11 WIN 55,212-2 does not significantly inhibit the PGE_2 sensitisation of the area of the capsaicin-evoked calcium transient.

(Same as figure 3.10 except that AreaRatio 6/5 is being examined)

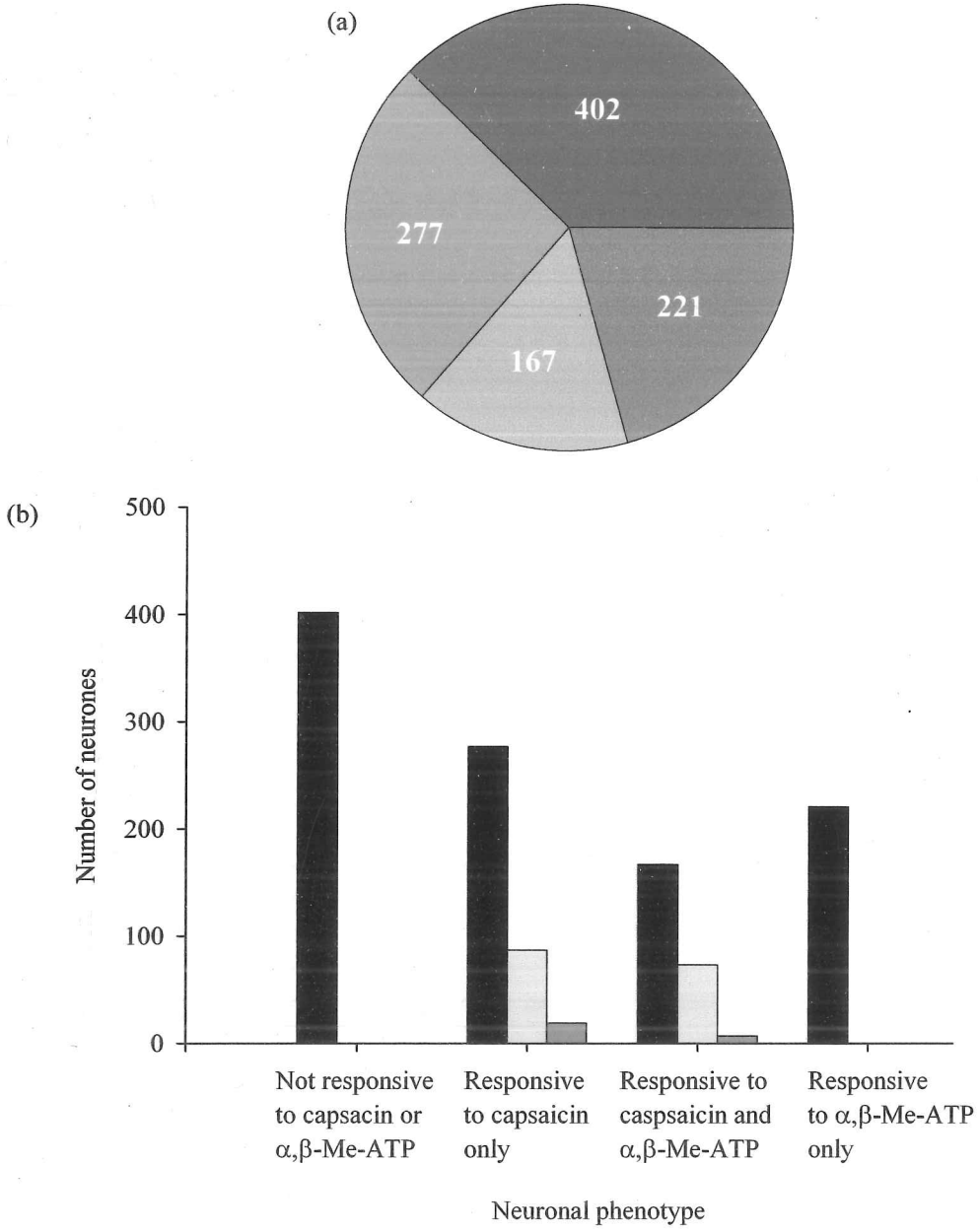

Figure 3.12 Characterisation of DRG neurones treated with PGE_2 and WIN 55,212-2.

(As figure 3.8 but with reference to figure 3.10(a))

sensitised neurones given the range of criteria laid out in Chapter 2 for selecting the population of neurones which should be assessed for sensitisation.

A comparison of the mean value for Ratio 6/5 and AreaRatio 6/5 of those neurones that had fallen above the upper confidence limit in control experiments, and sensitised in PGE2 only, PGE2 & WIN 55,212-2, and WIN 55,212-2 experiments failed to reveal any significant differences between these groups.

Conclusion

In summary, it can be seen that the activation of cannabinoid receptors on cultured DRG neurones by a two minute application of 100 nM WIN 55,212-2 does not significantly suppress the responsiveness of nociceptive neurones to capsaicin nor does it significantly suppress the responsiveness of nociceptive neurones sensitised by PGE_2.

The lack of an effect of WIN 55,212-2 in the present circumstances, given that in other circumstances the activation of cannabinoid receptors has been shown to inhibit voltage-gated calcium channel currents (Ross *et al.*, 2001) and TRPV1 mediated increases in intracellular calcium (Millns *et al.*, 2001), may be as a result of the nature of the technique used. Millns *et al.* (2001), who also used calcium imaging, employed a methodology whereby the cannabinoid was added between the first and second capsaicin additions, where some desensitisation could be expected to be seen on its own anyway. Ross *et al.* (2001) used the patch clamp method to provide a reading of the current flowing through voltage-gated calcium channels, and this could be expected to offer greater precision than calcium imaging in examining any effect of cannabinoid receptor activation. It must also be remembered that more than one component is involved in the generation of the calcium transient, and the Ross

et al. (2001) study was looking specifically at voltage-gated calcium channels. The technique used in the present study is such that desensitisation as a direct result of capsaicin addition is expected to be minimised. Furthermore, the nature of the analysis resulted in a rather small range in which neurones could be classified as desensitised. Thus, any assessment of the effect of cannabinoid receptor activation, while perhaps being more accurate than the Millns *et al.* (2001) study, may have been hindered both by the coarse nature of the analysis and the involvement of multiple components.

The other theory investigated in the present study was whether the activation of cannabinoid receptors attenuated the sensitisation mediated by PGE_2. Such an effect could be expected given that cannabinoid receptors have been shown to couple to the inhibition of adenylate cyclase, and PGE_2, a well known sensitising agent, brings about its effects, partially, via the activation of adenylate cyclase and PKA. Thus a form of physiological 'antagonism' might be expected to occur. On examination, however, WIN 55,212-2 was not seen to abolish sensitisation by PGE_2 when the percentage of neurones sensitised was compared. This was both with respect to Ratio 6/5, which compares the height of the transient itself, as well as to AreaRatio 6/5. Given that some difference does occur, a greater *n* number may have helped to reduce the s.e.m. and thus produce some significance. If the extent of sensitisation occurring between those neurones treated with PGE_2, and PGE_2 and WIN 55,212-2 is compared by examining the mean of the Ratio 6/5 values of those sensitised, there is visibly a slightly greater tail to the PGE_2 data, compared with the PGE_2 and WIN 55,212-2 data. Again, however, there was no significant difference when a one-way ANOVA (with Bonferroni correction) was applied to the data.

Therefore, given the lack of any significant effect, it was decided to conclude this area of work and move onto a different area. Suggestions for future investigations in this area would

be to try a greater and/or longer treatment with a cannabinoid receptor agonist preceeding the application of PGE_2 in the hope that this might result in some significance.

Chapter 4

Results: SNSRs

Introduction

The motivation for the work in this chapter comes from Lembo *et al.* (2002) who reported that a novel family of G-protein coupled receptors (GPCRs) was present in small diameter sensory neurones, and named them sensory neurone-specific receptors (SNSRs). They noted that a substantial proportion of neurones expressing these receptors also expressed the vanilloid receptor (TRPV1) (56%) and that most bound the plant isolectin B4 (IB_4) (76%) which is characteristic of the non-peptidergic group of nociceptive neurones (Bennet *et al.*, 1998). Utilising a heterologous expression system, these receptors were found to be selectively activated by the 8-22 fragment of the bovine adrenal medulla peptide 22, BAM (8-22). In the report by Lembo *et al.* (2002) the physiological function of BAM (8-22) was noted as still open for investigation. Further study by Cao *et al.* (2003) and Grazzini *et al.* (2004) found that *in vivo* SNSR activation resulted in both nociception and hyperalgesia. A fuller understanding of the molecular mechanisms of pathways activated by the SNSRs may allow the development of compounds that could inhibit the activation of these receptors and thus the subsequent algesia. Cao *et al.* (2003) and Grazzini *et al.* (2004) found that the response to heat was enhanced by the activation of SNSRs. Given that TRPV1 is involved in mediating the response of neurones to heat (Tominaga *et al.*, 1998), examining how SNSR activation may affect the response of cultured dorsal root ganglion (DRG) neurones to TRPV1 agonists would provide a means of establishing a cellular mechanism of action for BAM (8-22). The protocol established in the last chapter, in which repeated additions of capsaicin are used to probe TRPV1 activity, provides a means by which the effect of a treatment on those neurones expressing TRPV1, and thus neurones known to be detectors of noxious stimuli (Caterina *et al.*, 1997), can be examined. This same model was used to probe the action of the SNSR agonist, BAM (8-22).

The exact form that this action takes will depend on the signalling pathway involved. In heterologous expression systems SNSR agonists were seen to cause a calcium release from subcellular stores that was not affected by pertussis toxin pre-treatment (Lembo *et al.*, 2002; Han *et al.*, 2002; Grazzini *et al.*, 2004) but was completely inhibited by a phospholipase C (PLC) inhibitor (Han *et al.*, 2002) suggesting that rat SNSR1 is a $G_{\alpha q}$ coupled-receptor (Grazzini *et al.*, 2004). Thus its actions in DRG neurones could be mediated by either (1,2-diacylglycerol) DAG activation of Protein Kinase C (PKC) or inositol 1,4,5-triphosphate (IP_3)-dependent release of calcium from intracellular stores. So far, there is no published work on the effect of SNSR activation of cultured DRG neurones.

Effect of BAM (8-22)

A four minute application of 5 μM BAM (8-22) between the fifth and sixth capsaicin additions resulted in a sensitisation of the sixth response in a subset of neurones (Figure 4.1). In order to assess more quantitatively the degree to which treatment with BAM (8-22) resulted in a sensitisation of the response of neurones to capsaicin, control data were collected in the absence of drug treatment (Figure 4.2(a); black bars; n=90), and the 95% confidence limits for Ratio 6/5 were determined (0.076276 to 1.75834; Figure 4.2(a); red and blue dashed line, respectively). It should be noted that this represents the same control data as those presented in Chapter 3. The Ratio 6/5 data for neurones treated with BAM (8-22) (Figure 4.2(a); grey bars; n=373) were compared against the confidence limits determined from the control data. As the lower confidence limit is close to zero, an examination of the percentage of those neurones falling below the lower 95% confidence limit is of little value. However, if the Ratio 6/5 values for the control and BAM (8-22) treatment data are visually compared for

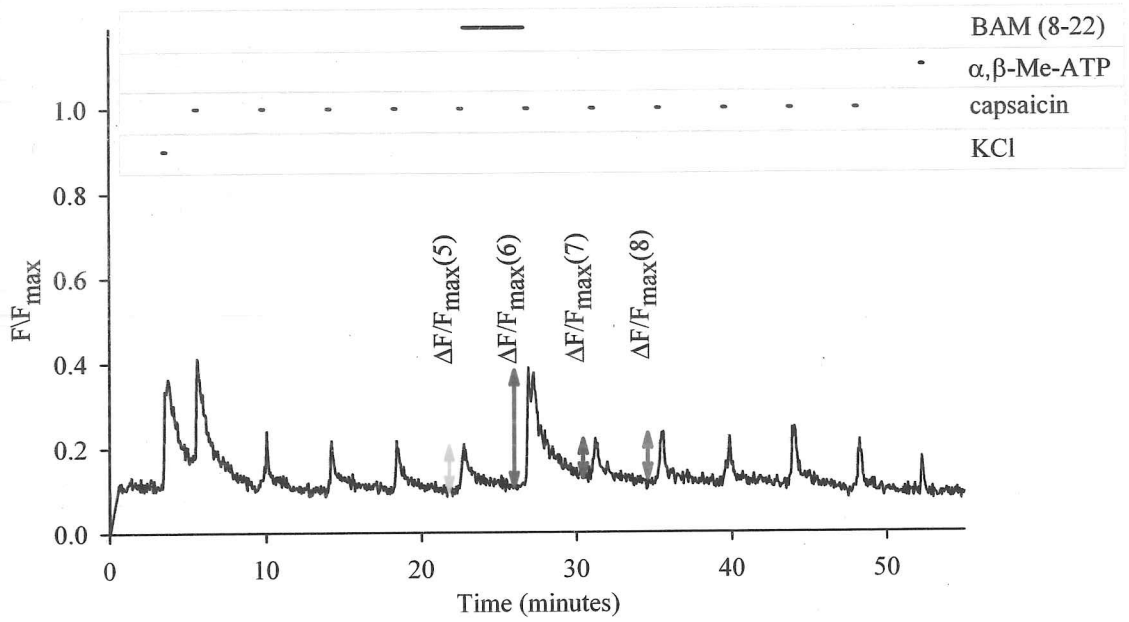

Figure 4.1 BAM (8-22) sensitises a cultured neonatal rat DRG neurone to capsaicin. The plot is of the intracellular calcium concentration of the neurone. For the duration of the time points indicated by bars the following solutions were added: 25 mM KCl, 100 nM capsaicin, 5 μM BAM (8-22) and 100 μM α,β-Me-ATP. The vertical lines with arrowheads adjacent to the calcium transients indicate the increases in intracellular calcium concentration evoked by the fifth, sixth, seventh and eighth capsaicin additions.

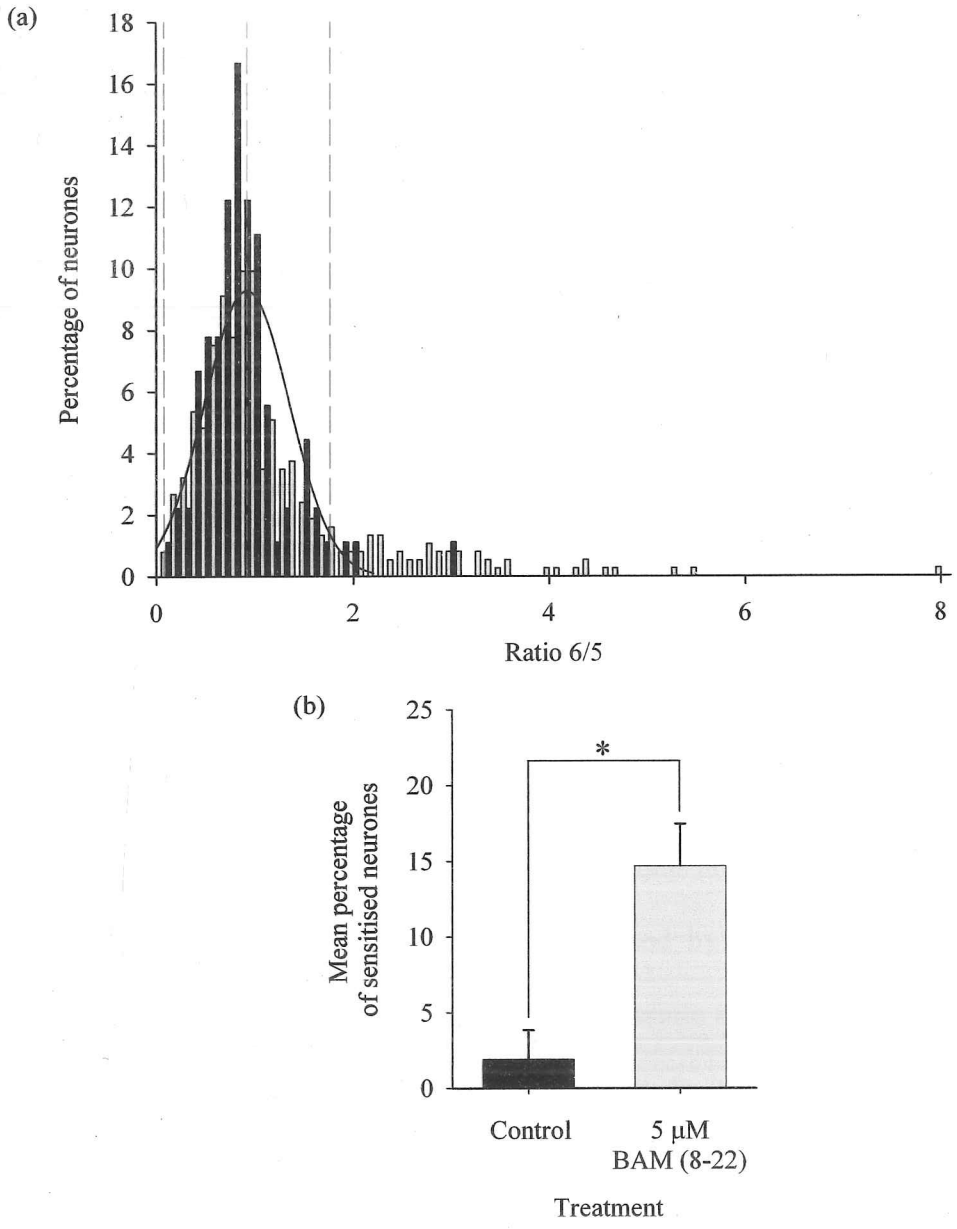

Figure 4.2 BAM (8-22) significantly sensitises DRG neurones to 100 nM capsaicin.

(a) A histogram displaying the percentage of neurones from control (black, *n*=90) and treatment (grey, *n*=373) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth capsaicin-evoked calcium transient (Ratio 6/5). The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (n_{exp}=13) and treatment (n_{exp}=19) experiments where the value of Ratio 6/5 is greater than the upper 95% confidence limit in figure (a). * indicates that the mean difference is significant at the 0.05 level.

values less than one, the results are fairly congruent suggesting no desensitising effect. If a neurone had a ratio that was above the upper 95% confidence limit, it was deemed to have been sensitised. The mean percentages (and the standard errors of the means) of neurones sensitised (Control=1.92±1.92%; 5 μM BAM (8-22)=14.70±2.78%; Figure 4.2(b)) were obtained for each set of coverslips (Control n_{exp}=13; 5 μM BAM (8-22) n_{exp}=19), and there was a significant difference at the 0.05 level between the control and BAM (8-22) data. In addition, the ratio of the area under the transients evoked by the fifth and sixth capsaicin additions was calculated (AreaRatio 6/5) (Figure 4.3(a)). There was, however, no significant difference at the 0.05 level with this measurement between the control data (4.37±2.41%) and the BAM (8-22) data (17.92±3.31%) (Figure 4.3(b)). Thus the application of BAM (8-22) for four minutes results in a significant proportion of neurones being sensitised in their response to capsaicin when the height of the evoked transients are compared.

The duration of this sensitisation was assessed by examining the ratios of the calcium transients evoked by the seventh and eighth capsaicin additions to the fifth capsaicin addition (Figure 4.4). The Ratio 7/5 and Ratio 8/5 values for the BAM (8-22) experiments were not significantly greater than these ratios for control experiments. Thus the sensitisation is short-lived and disappears within 4 minutes of application of BAM (8-22).

As with the PGE_2 experiments, the phenotype of the neurones present in these experiments was assessed in order to determine whether the subset of neurones sensitised by BAM (8-22) differed from either the general population of neurones or the subset of neurones which repeatedly responded to capsaicin. Figure 4.5(a) displays the number of neurones falling into each category for all of the BAM (8-22) experiments, and Figure 4.5(b) provides a comparison amongst all neurones (black), those that met the criteria laid out in Chapter 2 for a stable and noise-free repeated capsaicin response (light grey), and those sensitised by BAM (8-22) (dark grey). From Figure 4.5(b) it can be seen that for these experiments neurones

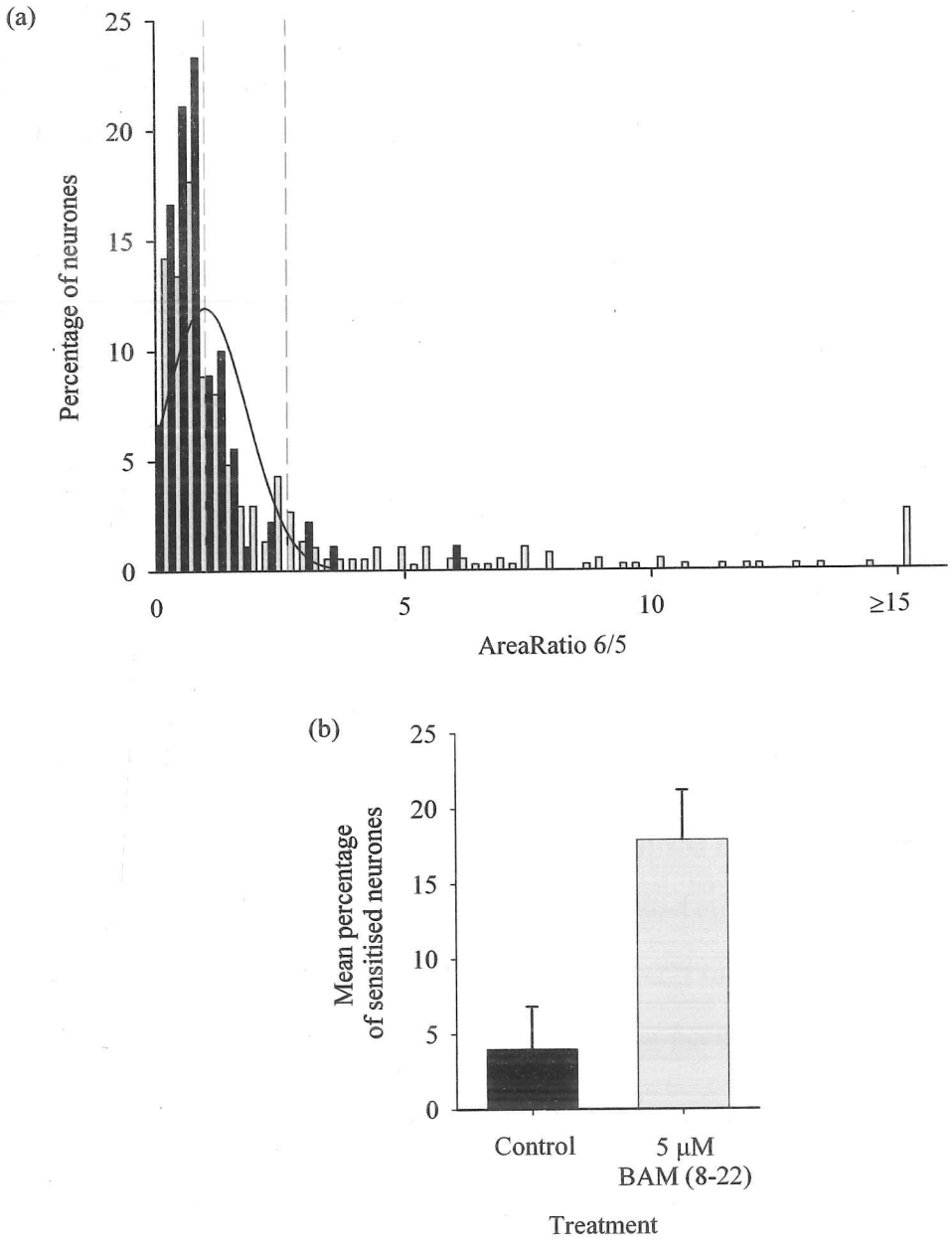

Figure 4.3 BAM (8-22) does not significantly increase the area of the capsaicin-evoked calcium transient.

(Same as figure 4.2 except that AreaRatio 6/5 is being examined)

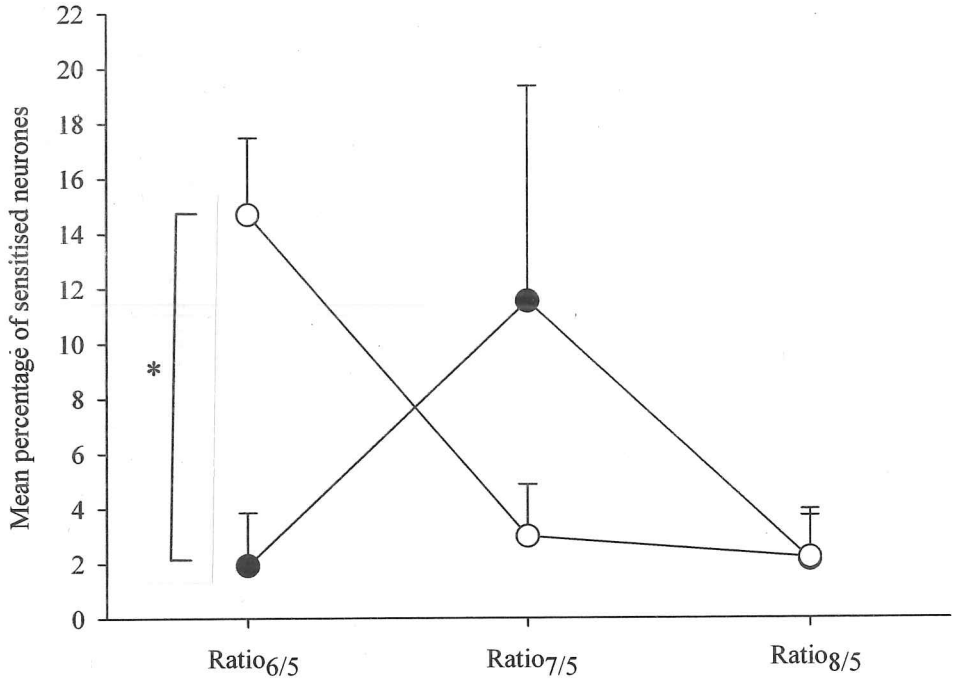

Figure 4.4 Sensitisation by BAM (8-22) is restricted to the subsequent capsaicin addition.

Plot of the mean percentage (with s.e.m.) of sensitised neurones identified by $Ratio_{6/5}$, $Ratio_{7/5}$, and $Ratio_{8/5}$ in control experiments (filled circles; n_{exp}=13,13,12) and experiments with a four minute treatment with 5 μM BAM (8-22) (open circles; n_{exp}=19,19,19).

* indicates that the mean difference is significant at the 0.05 level.

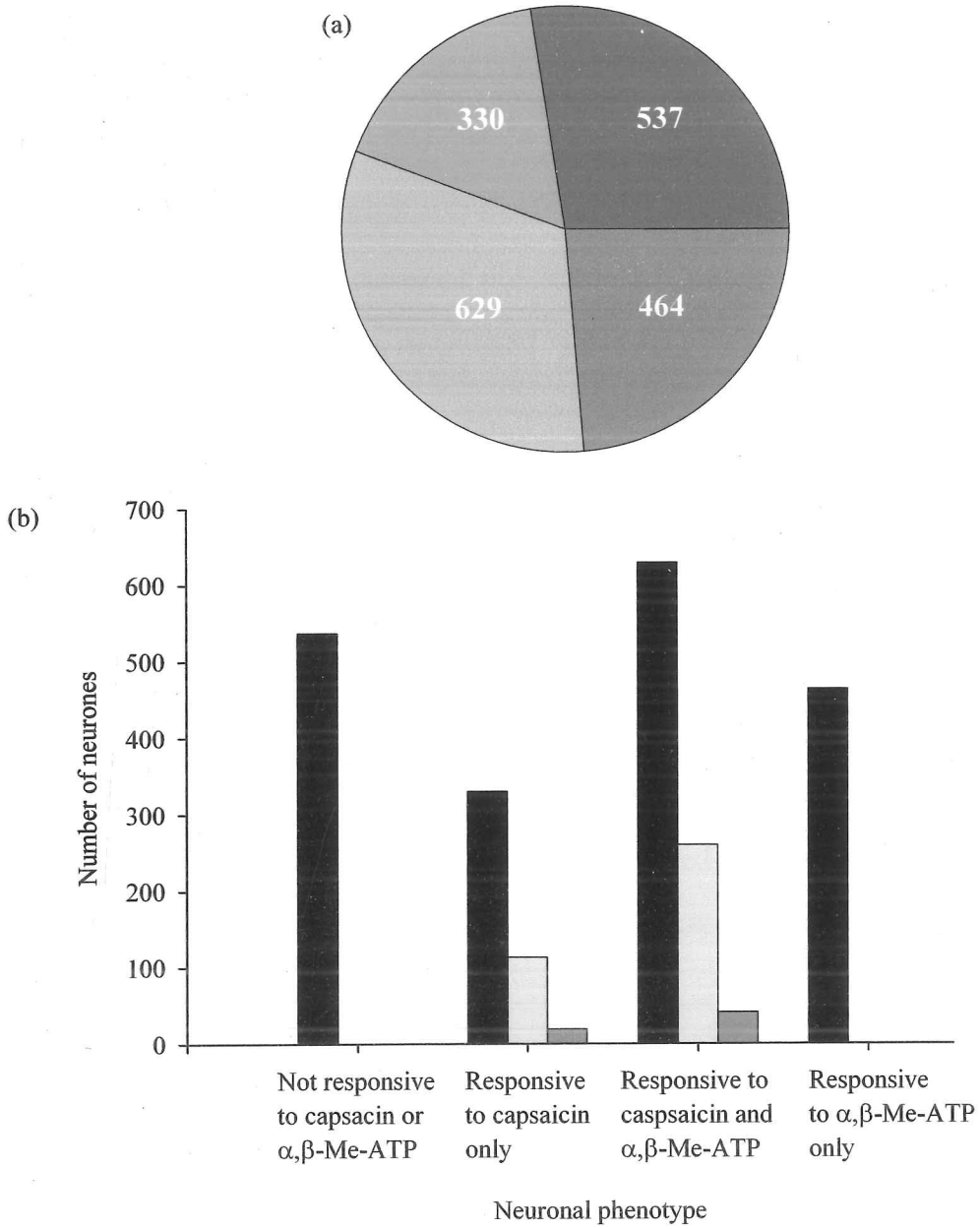

Figure 4.5 Characterisation of DRG neurones treated with BAM (8-22).

(a) A pie chart displaying the number of neurones analysed that responded to neither 100 nM capsaicin nor 100 μM α,β-Me-ATP (red), to only capsaicin (orange), to both capsaicin and α,β-Me-ATP (yellow), and to only α,β-Me-ATP (green). (b) A bar chart displaying the same information as in (a) (black), and in addition, displaying the number of neurones in each category for the subset of neurones that gave a stable response (light grey), and for the neurones of this subset that had a Ratio 6/5 value greater than the upper 95% confidence limit denoted in figure 4.2(a) (dark grey).

repeatedly responding to capsaicin are significantly more likely (Chi-square test; χ^2=30.14; $p \leq 0.001$) to respond to α,β-Me-ATP than to be non-responsive to it. Such a distribution was not seen in these neurones when sensitisation by PGE_2 was examined, and highlights the importance of assessing the neuronal population for each treatment before examining the distribution of those neurones that were sensitised. Furthermore, this distribution is maintained amongst those neurones sensitising to BAM (8-22) (Chi-square test; χ^2=4.17; $p \leq 0.05$).

Figure 4.6 displays a cultured DRG neurone that is a correlate of the 'silent' nociceptors and became responsive in the presence of BAM (8-22). In experiments where BAM (8-22) is applied for four minutes 5.10% of neurones were classified as 'silent' nociceptors (see Materials and Methods section for classification criteria) compared with 0.81% of neurones in control experiments. These neurones were not included when generating a histogram (e.g. Figure 4.2) as mathematically they would give a Ratio 6/5 value of infinity.

Protein kinase involvement in sensitisation

Previous studies with other sensitising compounds have revealed the involvement of protein kinases in mediating these effects at the cellular level. PGE_2, the sensitising inflammatory mediator discussed in the previous chapter, has been shown to activate both the cyclic 3',5'-adenosine monophosphate/Protein Kinase A (cAMP/PKA) pathway (Taiwo *et al.*, 1989; Pitchford and Levine, 1991; Taiwo and Levine, 1991; Cui and Nicol, 1995) and the Protein Kinase C (PKC) pathway (personal communication, V. Vellani). Therefore, in order to examine whether protein kinases may play a role in mediating the effect of BAM (8-22), the

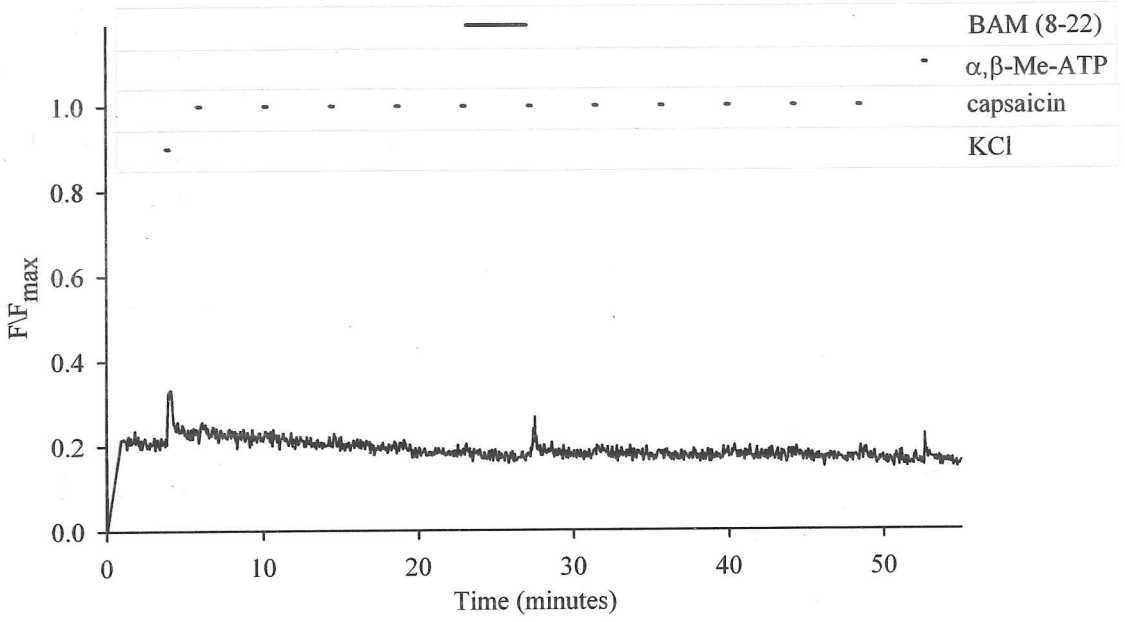

Figure 4.6 Example plot of BAM (8-22) sensitising a previously non-responsive neurone. The plot is of the intracellular calcium concentration of the neurone (as measured by F/F_{max}) over time. For the duration of the time points indicated by bars the following solutions were added: 25 mM KCl, 100 nM capsaicin, 5 μM BAM (8-22) and 100 μM α,β-Me-ATP.

general kinase inhibitor staurosporine (Ruegg & Burgess, 1989; Obreja *et al.*, 2002) was employed.

Effect of BAM (8-22) in the presence of staurosporine

200 nM staurosporine (Chen & Penington, 1996) was present throughout the experiments and the effect of a 4 minute application of 5 μM BAM (8-22) (grey; n=60; Figure 4.7(a)) was compared with control experiments (black; n=90; Figure 4.7(a)). The mean percentages (and the standard errors of the means) of neurones sensitised (Control=1.92±1.92%; 5 μM BAM (8-22) and 200 nM staurosporine=5.33±3.69%; Figure 4.7(b)) were obtained for each set of coverslips (Control n_{exp}=13; 5 μM BAM (8-22) and 200 nM staurosporine n_{exp}=10). These were not significantly different. Thus the presence of staurosporine attenuates the sensitising effect of BAM (8-22).

Effect of BAM (8-22) in the presence of Ro-31-8220

Because staurosporine is a general kinase inhibitor, other more selective kinase inhibitors were used in order to pinpoint the specific kinase involved. In the presence of 500 nM of the PKC-selective kinase inhibitor Ro-31-8220 (Davis *et al.*, 1992), the effect of a 4 minute application of 5 μM BAM (8-22) (grey; n=105; Figure 4.8(a)) was compared with a control population (black; n=90; Figure 4.8(a)). The mean percentages (and the standard errors of the means) of neurones sensitised (Control=1.92±1.92%; 5 μM BAM (8-22) and 500 nM Ro-31-8220=4.13±1.58%; Figure 4.8(b)) were obtained for each set of coverslips (Control n_{exp}=13; 5 μM BAM (8-22) and 500 nM Ro-31-8220 n_{exp}=13). These were not significantly different, and, furthermore, in the presence of Ro-31-8220, the response was significantly different from

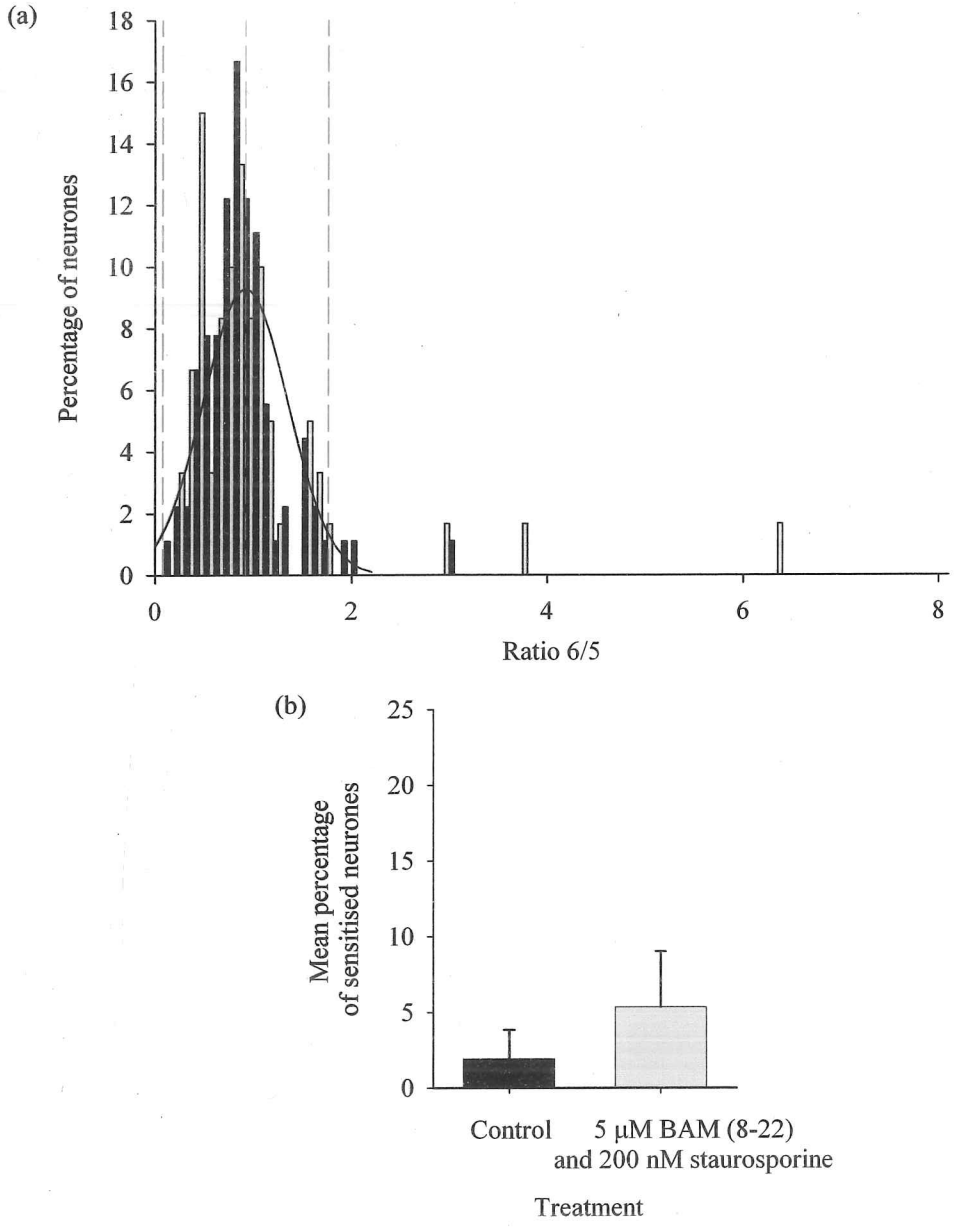

Figure 4.7 Staurosporine does not significantly inhibit sensitisation by BAM (8-22).

(a) A histogram displaying the percentage of neurones from control (black, *n*=90) and treatment (grey, *n*=60) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth transient (Ratio 6/5). The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (n_{exp}=13) and treatment (n_{exp}=10) experiments where the value of Ratio 6/5 for the neurones is greater than the upper 95% confidence limit denoted in figure (a).

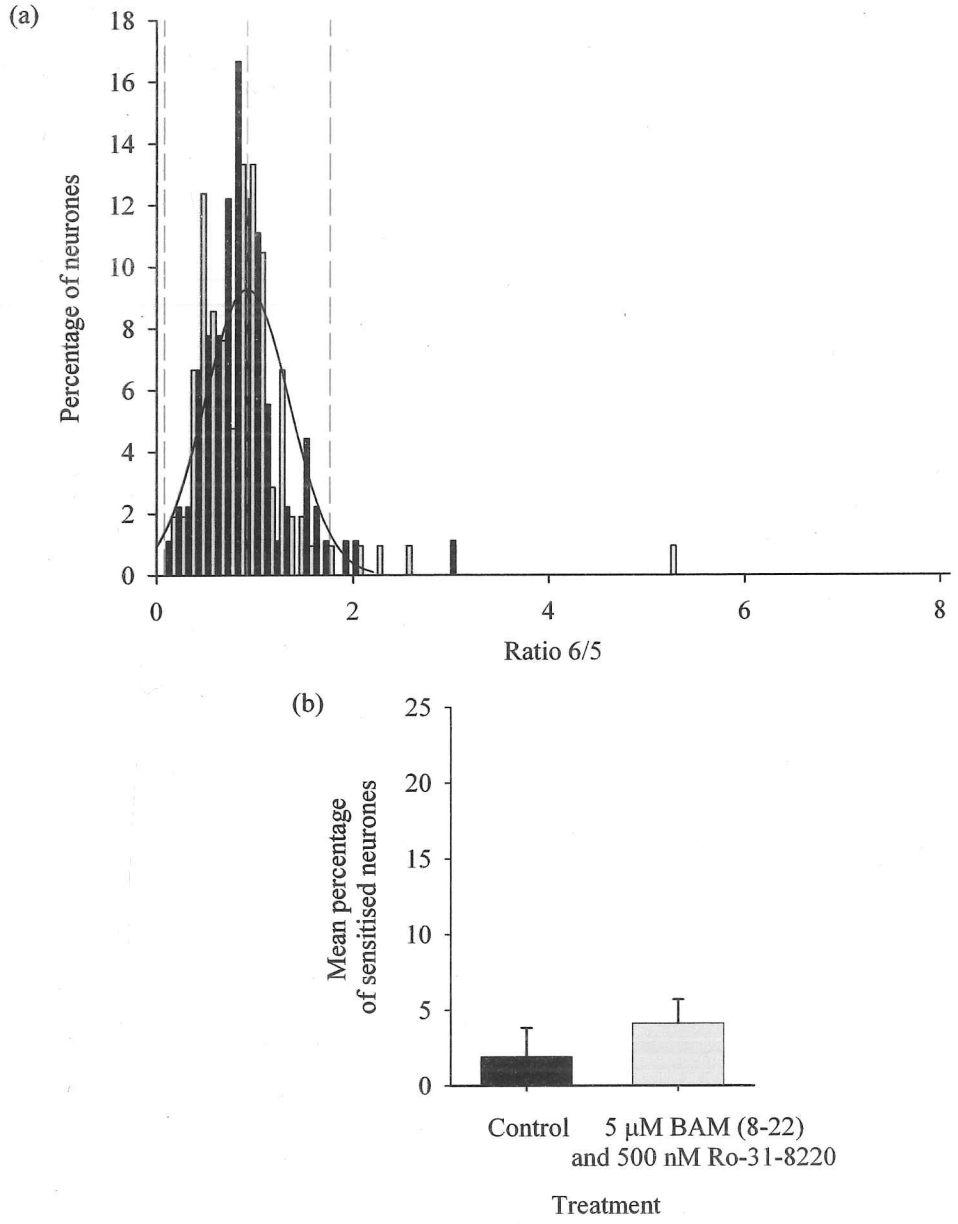

Figure 4.8 Ro-31-8220, a PKC selective inhibitor, significantly inhibits the sensitisation by BAM (8-22).

(a) A histogram displaying the percentage of neurones from control (black, *n*=90) and treatment (grey, *n*=105) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth transient (Ratio 6/5). The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (nexp=13) and treatment (nexp=13) experiments where the value of Ratio 6/5 is greater than the upper 95% confidence limit in figure (a).

BAM (8-22) alone. In addition, the ratio of the area under the transients evoked by the fifth and sixth capsaicin additions was calculated (AreaRatio 6/5) (Figure 4.9(a)). There was no significant difference at the 0.05 level between the control data (4.37±2.41%) and the BAM (8-22) and Ro-31-8220 data (14.14±7.67%) (Figure 4.9(b)). Thus the presence of Ro-31-8220 attenuates the sensitising effect of BAM (8-22).

Effect of BAM (8-22) in the presence of H-89

In the presence of 500 nM of the PKA-selective kinase inhibitor H-89 (Shu & Mendell, 2001), the effect of a 4 minute application of 5 μM BAM (8-22) (grey; n=118; Figure 4.10(a)) was compared with a control population (black; n=90; Figure 4.10(a)). The mean percentages (and the standard errors of the means) of neurones sensitised (Control=1.92±1.92%; 5 μM BAM (8-22) and 500 nM H-89=10.49±3.19%; Figure 4.10(b)) were obtained for each set of coverslips (Control n_{exp}=13; 5 μM BAM (8-22) and 500 nM H-89 n_{exp}=13). These were not significantly different. In addition, the ratio of the area under the transients evoked by the fifth and sixth capsaicin additions was calculated (AreaRatio 6/5) (Figure 4.11(a)). There was no significant difference at the 0.05 level between the control data (4.37±2.41%) and the BAM (8-22) and H-89 data (13.32±4.01%) (Figure 4.11(b)).

Summary of Calcium Imaging Experiments

Figure 4.12 summarises the results obtained from the calcium imaging experiments with the selective SNSR agonist BAM (8-22). The application of 5 μM BAM (8-22) for 4 minutes

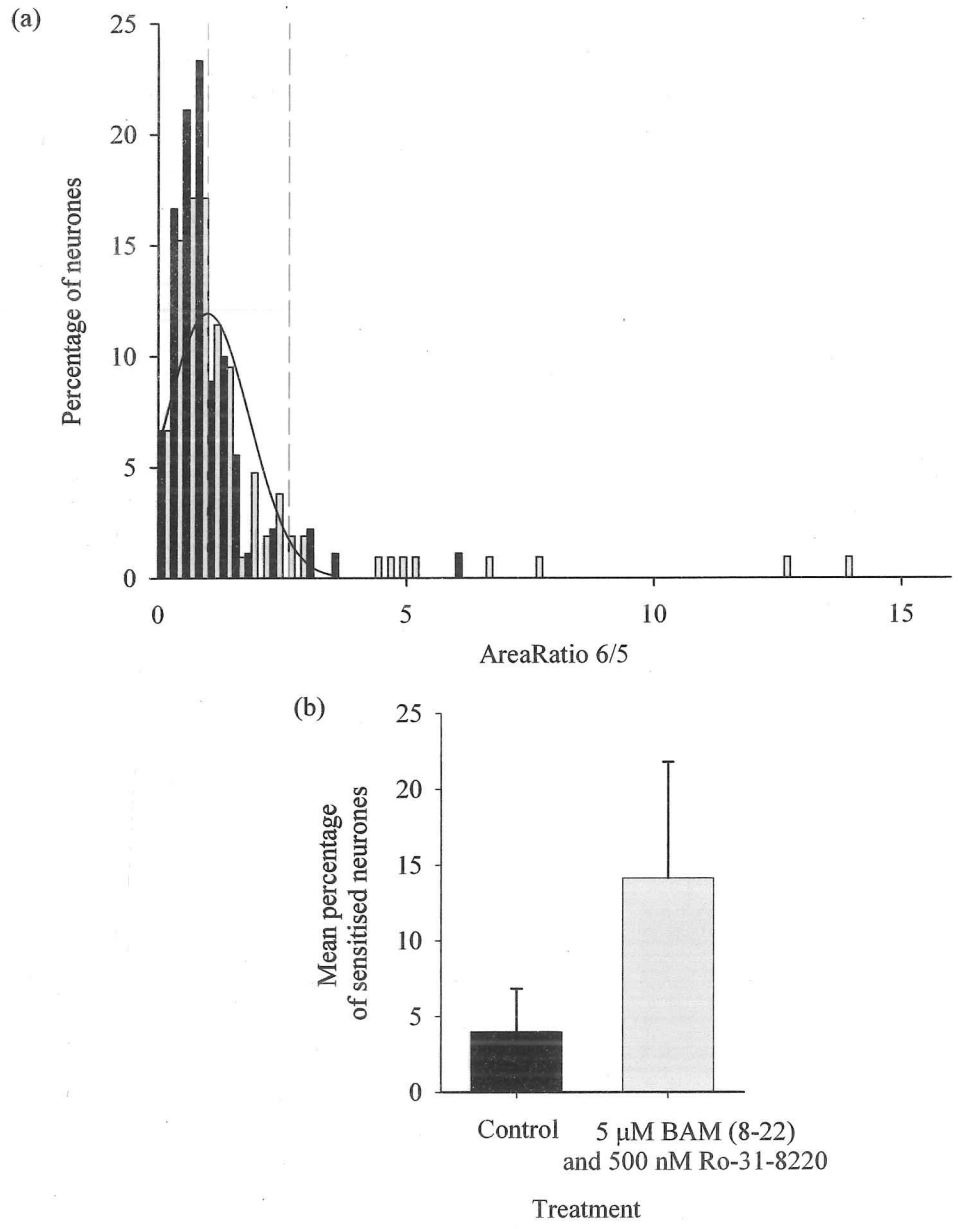

Figure 4.9 Ro-31-8220, a selective PKC inhibitor, does not significantly attenuate the increase in area of the capsaicin-evoked calcium transient mediated by BAM (8-22).

(Same as figure 4.8 except that AreaRatio 6/5 is being examined)

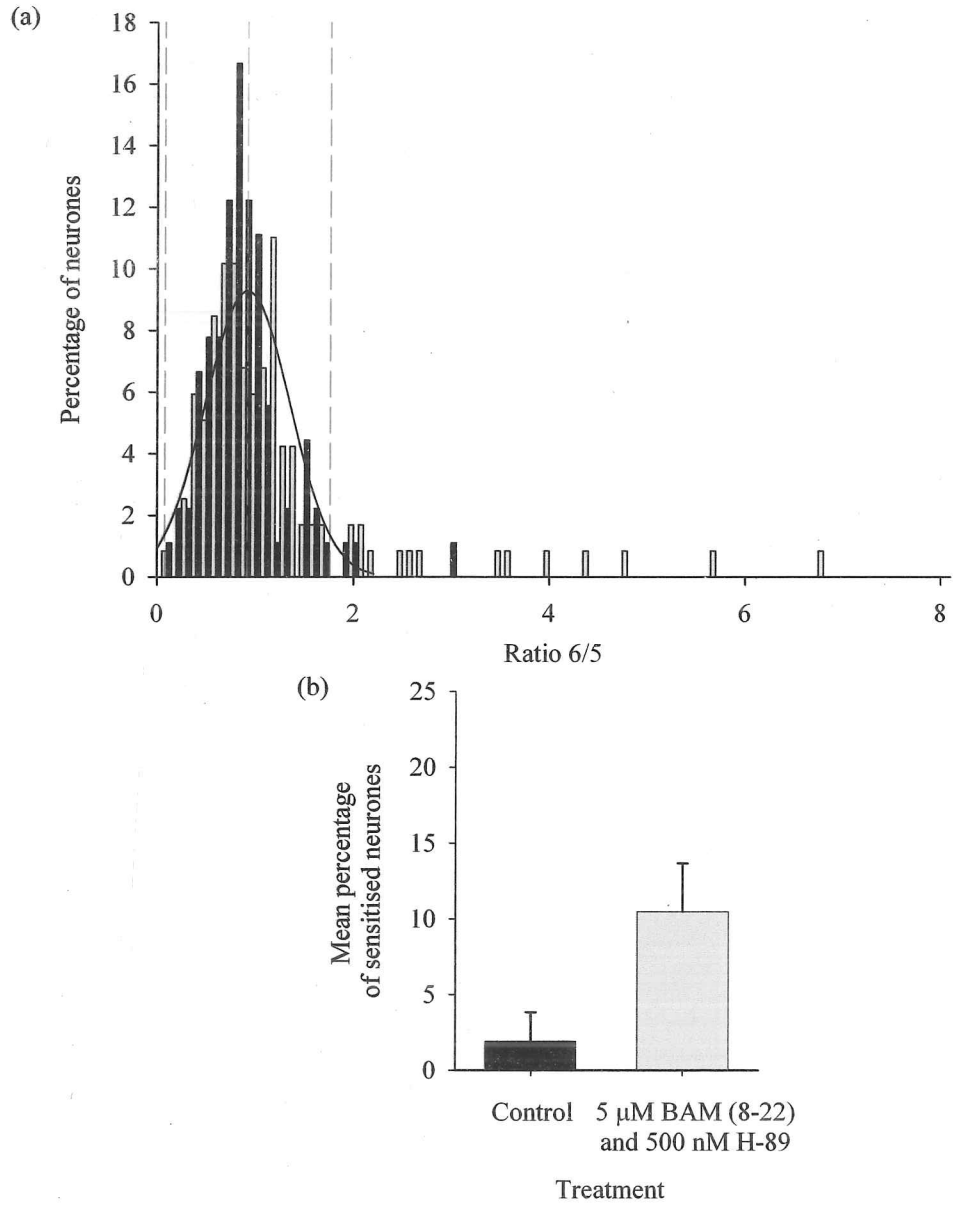

Figure 4.10 H-89, a PKA selective inhibitor, does not significantly inhibit the sensitisation by BAM (8-22) of the response of DRG neurones to capsaicin.

(a) A histogram displaying the percentage of neurones from control (black, *n*=90) and treatment (grey, *n*=118) experiments in each range of ratios comparing the size of the sixth capsaicin-evoked calcium transient against the size of the fifth transient (Ratio 6/5). The single black line plot denotes the Gaussian distribution fitted to the control data, with the vertical dashed lines denoting the lower 95% confidence limit (red), the mean (green), and the upper 95% confidence limit (blue) of this distribution. (b) A bar chart displaying the mean percentage of neurones with s.e.m. in control (n_{exp}=13) and treatment (n_{exp}=13) experiments where the value of Ratio 6/5 for the neurones is greater than the lower 95% confidence limit denoted in figure (a).

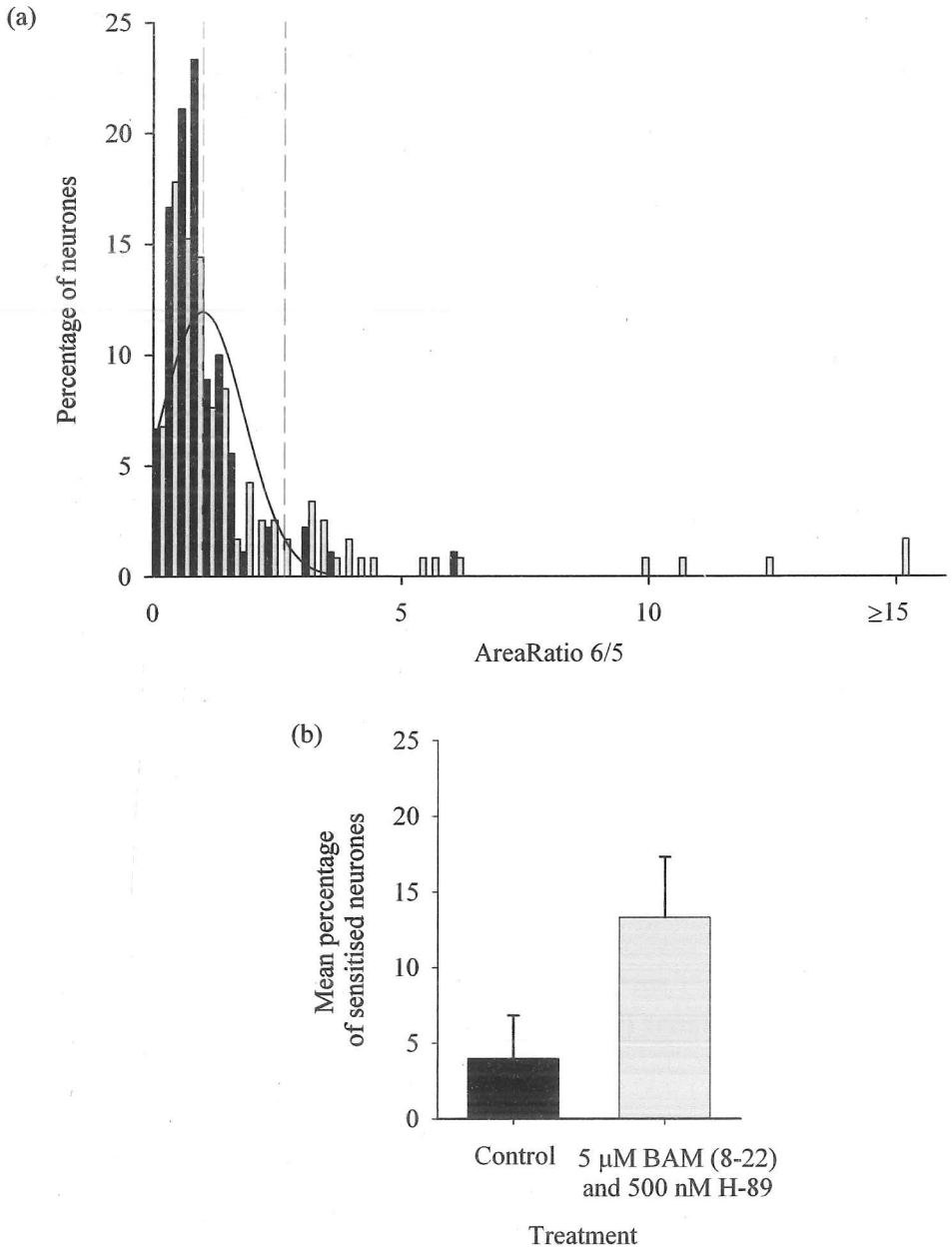

Figure 4.11 H-89, a PKA selective antagonist, does not significantly attenuate the increase in area of the capsaicin-evoked calcium transient mediated by BAM (8-22). (Same as figure 4.10 except that AreaRatio 6/5 is being examined).

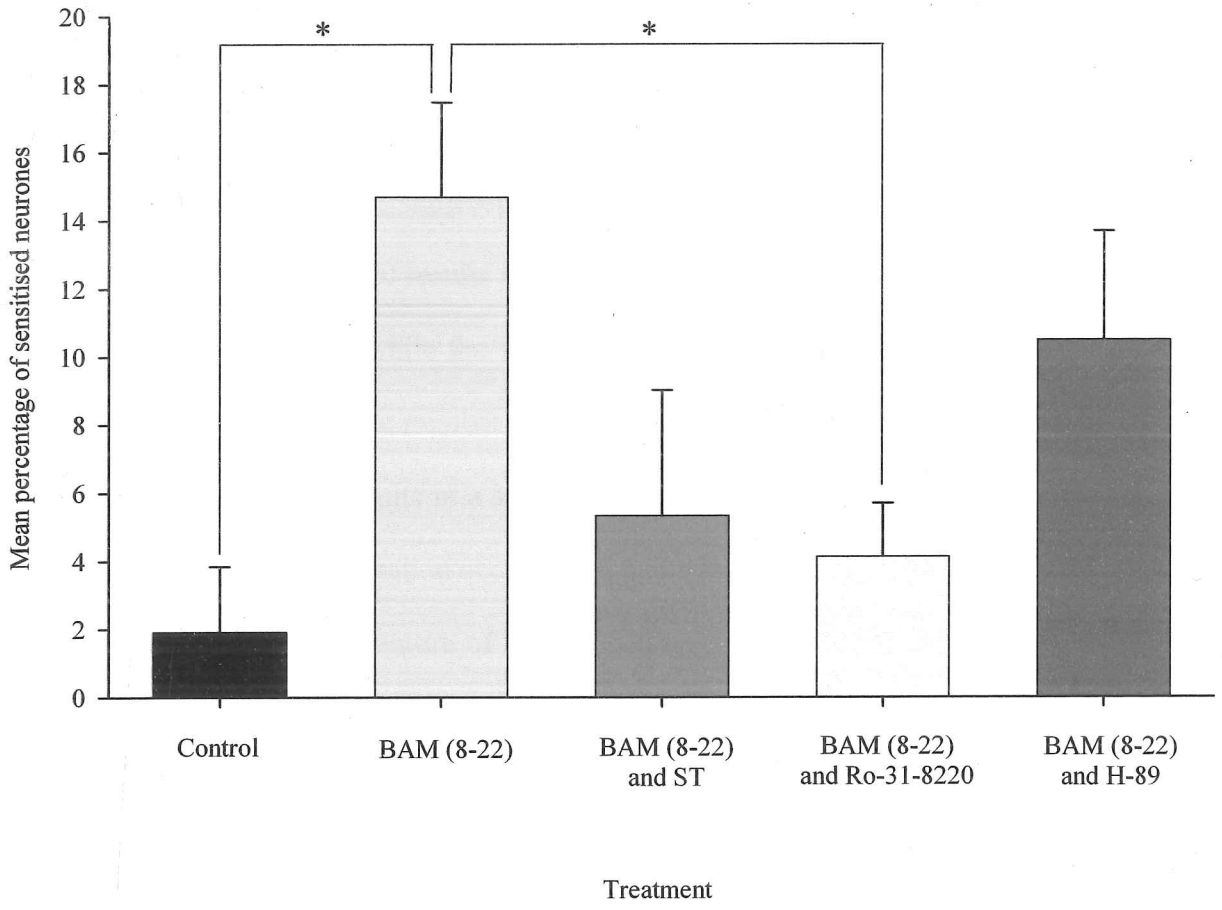

Figure 4.12 Summary Bar Chart: BAM (8-22) sensitises the response of neurones to capsaicin via a mechanism involving PKC.

Bar chart summarising the sensitisation evident from Ratio 6/5 in the experiments with BAM (8-22) and various protein kinase inhibitors. Each bar represents the mean percentage of neurones (with s.e.m.) where the value of Ratio 6/5 for the neurones is greater than the upper 95% confidence limit for the control data. Control (n_{exp}=13), 5 μM BAM (8-22) (nexp=19), and 5 μM BAM (8-22) with the following inhibitors: 200 nM staurosporine (ST) (nexp=10), 500 nM Ro-31-8220 (nexp=13), and 500 nM H-89 (nexp=13). * indicates that the mean difference is significant at the 0.05 level when compared using a one-way analysis of variance with a Bonferroni post hoc test.

between the fifth and sixth capsaicin additions results in a significant number of neurones being sensitised compared with control. The general kinase inhibitor staurosporine results in a marked, but not significant, suppression of the number of neurones sensitised. The PKC-selective inhibitor Ro-31-8220 results in a significant suppression of the number of neurones sensitised by BAM (8-22), while the PKA-selective inhibitor H-89 causes a minor, but not significant, suppression of the number of neurones sensitised by BAM (8-22). Therefore, it appears that BAM (8-22) results in a sensitisation of the response of nociceptive neurones to capsaicin and that this sensitisation occurs via a pathway involving PKC.

Some further ideas of the nature of this sensitisation are given by other analysis that was carried out on the same data. When the AreaRatio 6/5 data was compared, BAM (8-22) may have increased the percentage of neurones falling above the upper 95% confidence limit, although the difference did not reach statistical significance. Furthermore, when Ro-31-8220 was present there was a decrease in the mean of the percentage of neurones sensitised but the s.e.m. in this case was rather large. The calcium imaging technique employed may well not provide an accurate enough means by which to assess the involvement of TRPV1, given the potential involvement of voltage-dependent calcium channels, and thus the utilisation of a more precise technique such as patch clamping is necessary. This would permit further assessment of the involvement of PKC.

Before establishing a direct involvement of TRPV1, and whether PKC is linked to sensitising it. The PKC isoforms involved in the sensitisation of nociceptive neurones by BAM (8-22) were examined. This was done by utilising an immunocytochemical assay which examined the translocation of various PKC isoforms.

Immunocytochemistry

Activation of PKC often leads to its translocation to the membrane. In cultured neonatal rat sensory neurones there are 5 known isoforms of PKC: βI, βII, δ, ε, and ζ (Cesare *et al.*, 1999a). The isoforms βI and βII are already associated with the cellular membrane and so it is not possible to examine their activation through a translocation assay. The isoforms δ, ε and ζ are not membrane-associated (Cesare *et al.*, 1999a) and thus can be assessed using this assay.

In addition to exposure to 5 μM BAM (8-22) for 4 minutes, neurones were exposed to DPBS only as a control treatment, and for δ and ε isoforms, were exposed to PMA as a known translocator of these isoforms of PKC. PKC- ζ is not activated by phorbol esters (Ways *et al.*, 1992). Figure 4.13 contains example images for the three different isoforms of PKC examined when the neurones had been treated with DPBS (Figure 4.13(a)), 5 μM BAM (8-22) (Figure 4.13(b)), and 1 μM PMA (Figure 4.13(c)). In none of the control experiments (Figure 4.13(a)) was any clear translocation to the periphery seen. Clear translocation was taken to be a solid ring of red adjacent to the plasma membrane. Such a clear translocation is evident in Figure 4.13(c) for the PMA data. When neurones treated with BAM (8-22) were examined no clear translocation to the periphery was seen (Figure 4.13(b)). Such an effect is reflected in the overall results presented in Table 4.1.

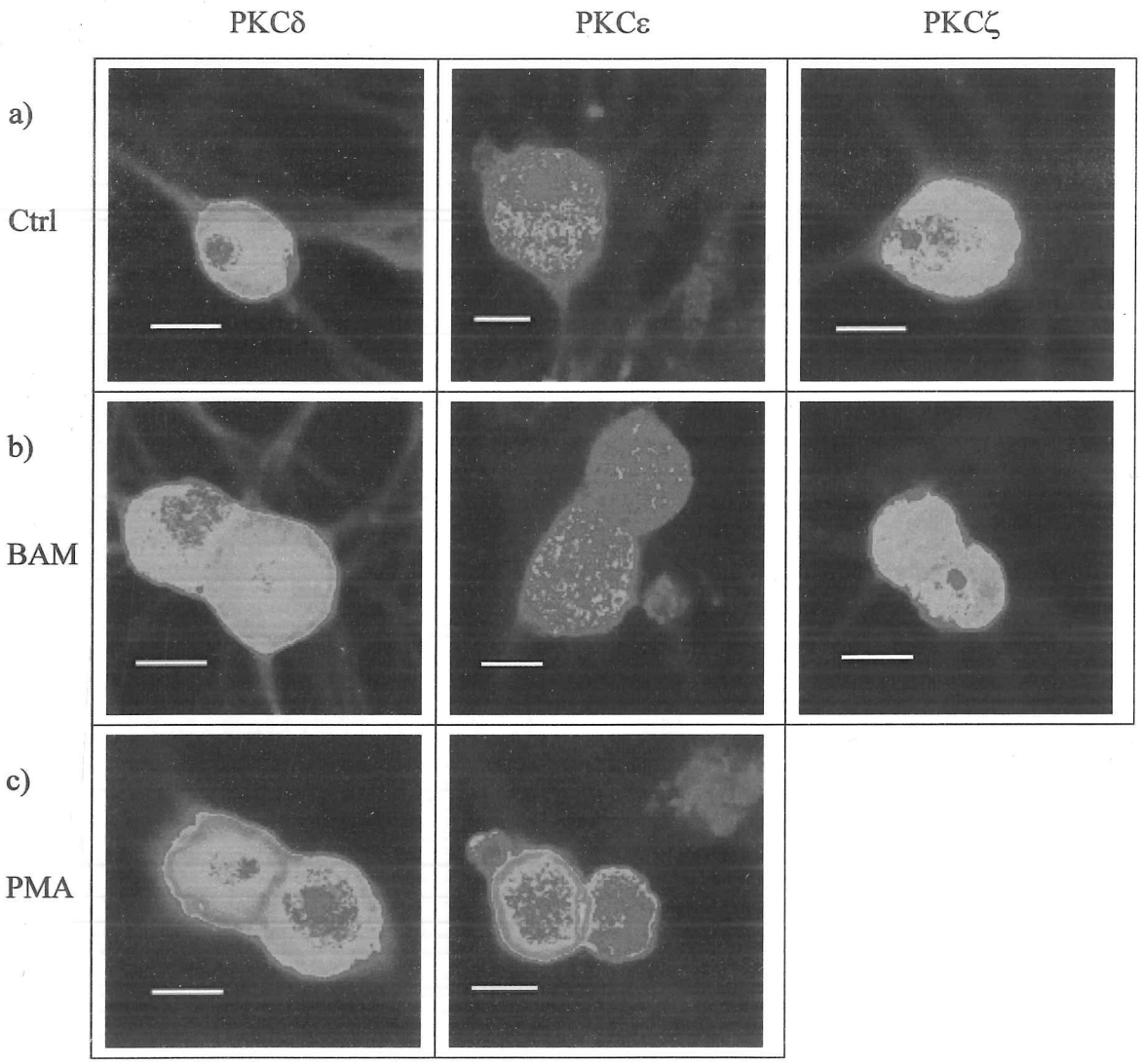

Figure 4.13 Localisation of Protein Kinase C isoforms in cultured DRG neurones

Neurones were exposed for four minutes to (a) PBS, (b) 5μM BAM (8-22) and (c) 1μM PMA. The scale bars in the example images above represent 10μm.

PKC isoform	Treatment	Number of neurones analysed	Number of neurones displaying translocation
Delta (δ)	DPBS	183	2
	1 μM PMA (4 min)	182	57
	5 μM BAM (8-22) (4 min)	362	5
Epsilon (ε)	DPBS	341	2
	1 μM PMA (1 min)	134	37
	1 μM PMA (4 min)	225	38
	5 μM BAM (8-22) (1 min)	445	0
	5 μM BAM (8-22) (4 min)	390	0
Zeta (ζ)	DPBS	172	0
	5 μM BAM (8-22) (4 min)	369	0

Table 4.1 Translocation of various PKC isoforms by PBS, PMA and BAM (8-22)

The delta (δ) isoform of PKC was seen to be significantly translocated (at the 0.01 level) by a 4 minute treatment with 1 μM PMA compared with control data, but was not translocated when BAM (8-22) was applied. The epsilon (ε) isoform of PKC has been previously identified as playing a role in mediating the effect of sensitising agents on nociceptive neurones (Cesare *et al.*, 1999a). The effect has been previously identified to be time dependent when the translocation is evoked by a receptor agonist (i.e. bradykinin) (Cesare *et al.*, 1999a) and so applications lasting 1 and 4 minutes were tried. 1 μM PMA, for either length of time, resulted in a significant percentage of neurones showing translocation (at the

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0.01 level) compared with control data where negligible translocation was seen. Treatment with 5 μM BAM (8-22) for either length of time did not result in any translocation. The zeta (ζ) isoform of PKC is not translocated by PMA and thus it was not possible to carry out a positive control for this isoform, however, there was no translocation with either the control or BAM (8-22) treatment.

Thus none of the three isoforms of PKC examined showed any translocation by BAM (8-22). This may be because one of the PKC isoforms (βI and βII) which are already associated with the plasma membrane is activated by BAM (8-22). Alternatively, other work has shown that PKC-δ can activate the non-receptor tyrosine kinase, Src (Brandt *et al.*, 2003). Src, in turn, can phosphorylate TRPV1 on tyrosine residues to potentiate its activity (Jin *et al.*, 2004). Therefore, a possible hypothesis may be the involvement of this pathway in the sensitisation of TRPV1 by BAM (8-22). Therefore, although BAM (8-22) did not result in any significant translocation of PKC-δ, it could still be acting via this isoform. Further work will need to be carried out to investigate this possibility.

Conclusion

Activation of SNSRs by the use of the selective ligand BAM (8-22) was found to sensitise the response of a subset of those neurones responding to capsaicin (and thus expected to express TRPV1). This provides a cellular basis for the behavioural studies reported by Cao *et al.* (2003) and Grazzini *et al.* (2004) in which BAM (8-22) was found to cause heat hyperalgesia. The sensitisation of the amplitude of the capsaicin-evoked calcium increase was found to be mediated by PKC. When the isoforms were examined using an immunocytochemical translocation assay, none of those tested (PKC-δ, ε and ζ) were found to

translocate in response to treatment with BAM (8-22). Further work is necessary in this area in order to elucidate fully the intracellular signalling pathway that underlies the sensitisation by BAM (8-22). The calcium imaging technique employed, whilst permitting a large sample of cells to be screened, does not, necessarily, give a clear indication of the involvement of TRPV1 because of the involvement of voltage-gated calcium channels in the generation of the capsaicin-evoked calcium transient (discussed further in Chapter 6). In order to further elucidate the mechanism of action it was decided to investigate if BAM (8-22) directly sensitised TRPV1 utilising the patch clamp method. Such an effect has been reported before for other sensitising agents such as PGE_2, and so is a strong possibility.

Chapter 5

Results: SNSRs and TRPV1

Introduction

In the last chapter, it was shown that the activation of sensory neurone-specific receptors (SNSRs) on a subset of nociceptive neurones resulted in a sensitisation of their response to capsaicin. Furthermore, this sensitisation was seen to be mediated by Protein Kinase C (PKC). Because in the calcium imaging experiments the neurones were not under voltage clamp, the sensitisation of the response to capsaicin could be a result of the modification of a number of cellular sites involved in setting the sensitisation of neuronal excitability (e.g. voltage-gated ion channels). Furthermore, the fact that it was the Ratio 6/5 values (a measure of the height of the evoked calcium transient and thus likely to be associated with the activation of voltage-gated calcium channels) that were substantially sensitised, and that the AreaRatio 6/5 values were sensitised in a non-significant manner, meant that further investigation was necessary in order to establish if a direct sensitisation of TRPV1 occurred. In order to examine if TRPV1 itself was sensitised as a result of the activation of SNSRs by BAM (8-22), it was decided to carry out experiments in which the membrane current activated by capsaicin was examined more directly under voltage clamp conditions. This was not tried initially given the greater experimental difficulty and the associated lower number of neurones assessed.

Repeated capsaicin additions

Cultured dorsal root ganglion (DRG) neurones were patch clamped in the whole-cell configuration, and held at a membrane potential of -80 mV. They were then exposed to 100 nM capsaicin for 1 s, every 30 s. The peak inward current evoked by the capsaicin

applications was measured. Figure 5.1(a) shows an example plot of a control experiment and Figure 5.1(b) displays individual capsaicin-evoked inward currents from the fifth and eighth capsaicin additions. The experimental protocol provided a suitable model whereby any modulation of TRPV1 could be examined.

The effect of BAM (8-22) on TRPV1

To examine the effect of BAM (8-22) on TRPV1, 5μM BAM (8-22) was applied for 5 minutes following the sixth capsaicin addition. Figure 5.2(a) shows an example plot of a neurone showing sensitisation by BAM (8-22) and Figure 5.2(b) displays individual capsaicin-evoked inward currents from the fifth and eighth capsaicin additions. Because of the variability in the consistency of the response of individual neurones to capsaicin there was some difficulty in finding an adequate means for quantatively assessing whether a neurone had been sensitised by the application of BAM (8-22). Figure 5.3 contains some examples of neurones that were not sensitised when exposed to BAM (8-22). In Figure 5.3(a) the addition of BAM (8-22) resulted in an initial increase in the inward current but this soon settled down to baseline. This initial increase is therefore likely to have been due to a movement artefact following the solution change and not a result of the agonist itself. Figure 5.3(b) displays a neurone where the addition of BAM (8-22) had no effect at all.

Therefore, the following method was used for analysis. When BAM (8-22) was added it was present from the sixth capsaicin addition until the fifteenth addition. Thus the three points prior to the BAM (8-22) treatment period, and all the points after, were used to fit a predicted baseline using linear regression. The differences between the points and this predicted baseline were calculated, and for each neurone 99.9% confidence limits were fitted

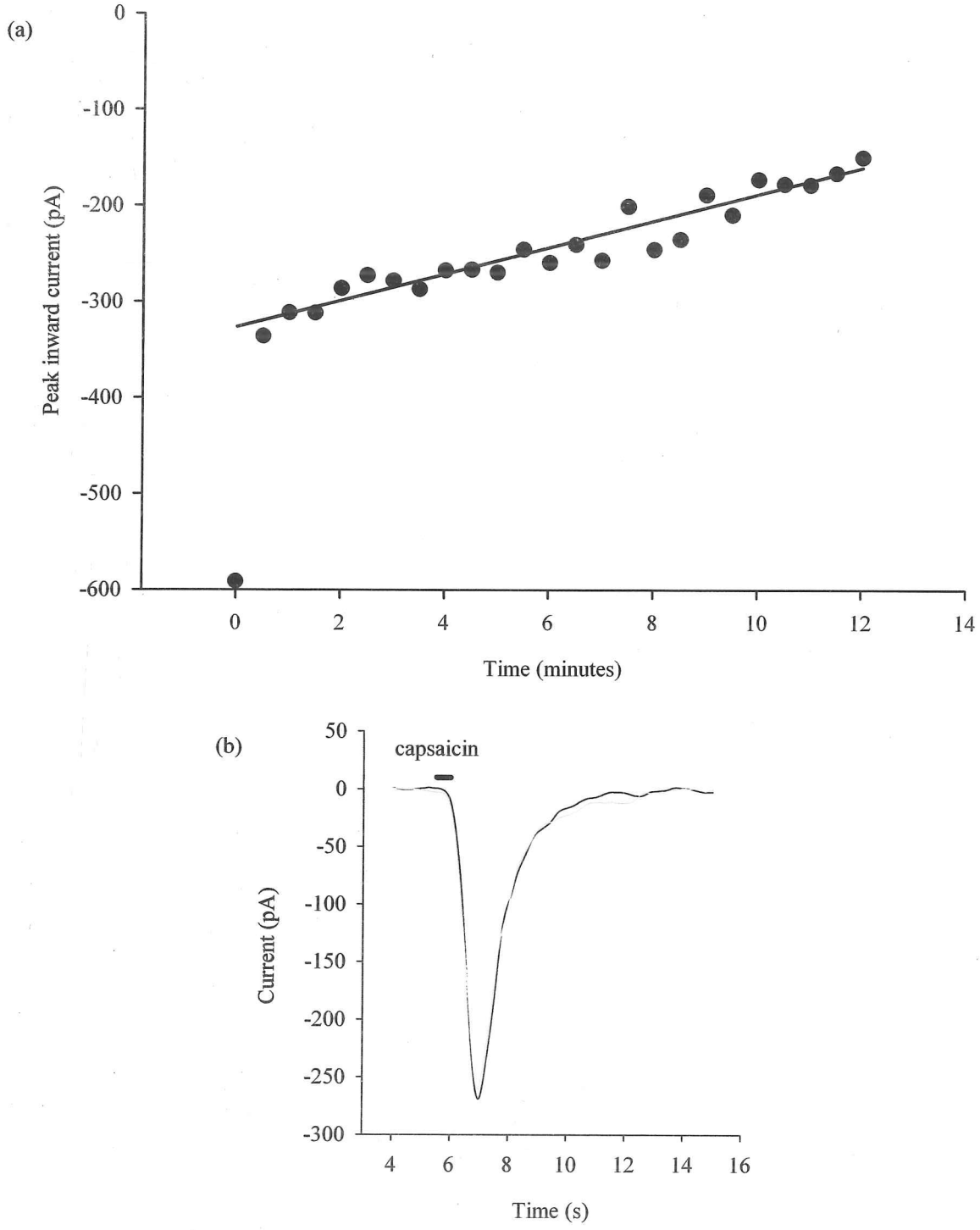

Figure 5.1 Example of a control neurone repeatedly responding to capsaicin.

(a) Responses to 100 nM capsaicin are denoted by the points on the graph and the predicted baseline by the black line. (b) Individual evoked responses (filtered at 1Hz) to 100 nM capsaicin from the neurone detailed in (a). The black trace is the response at 2 minutes, and the grey trace is the response at 3.5 minutes. The application of capsaicin is indicated by the black line.

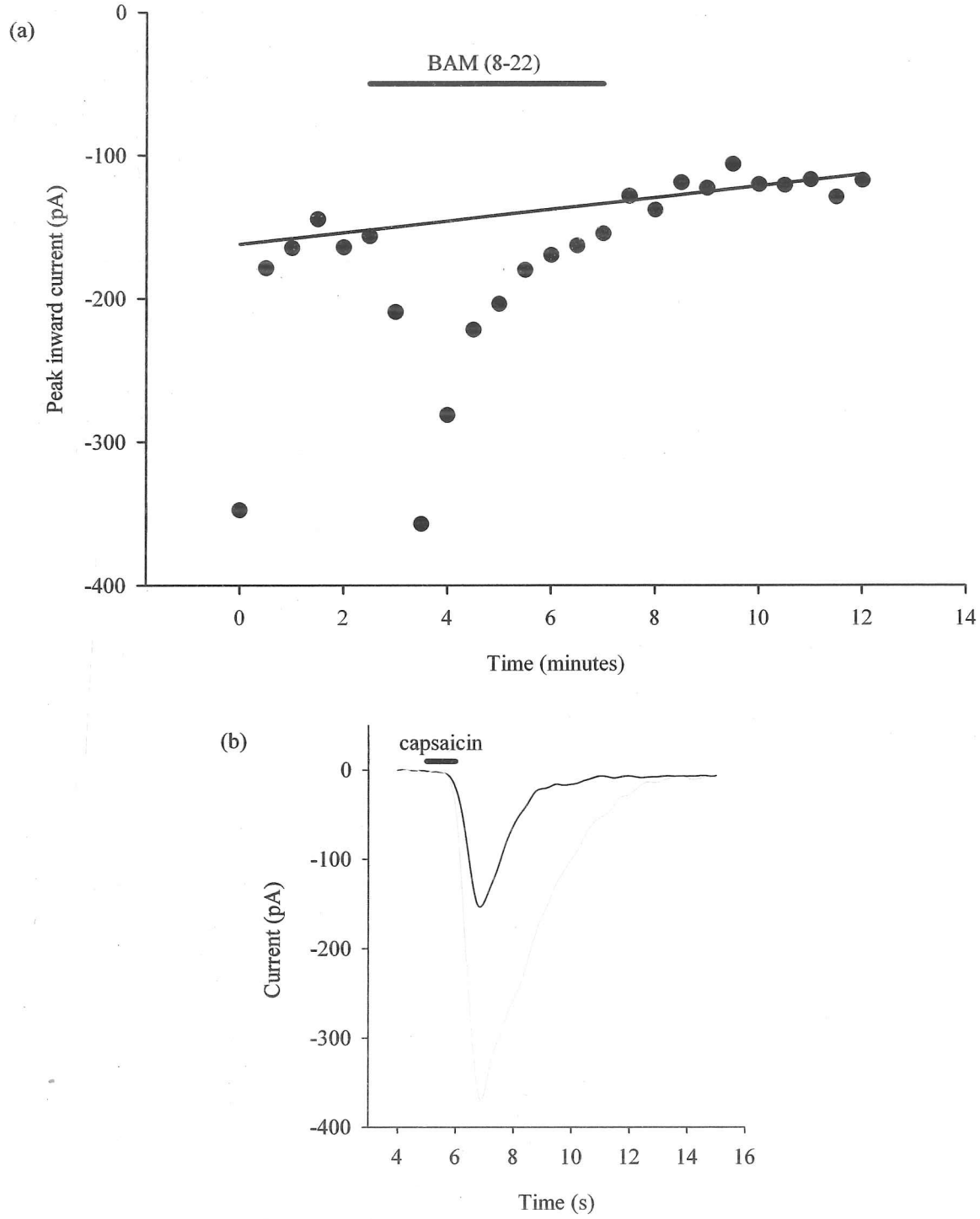

Figure 5.2 An example of BAM (8-22) sensitising the response of a neurone to capsaicin.

(As for figure 5.1 with the addition of 5 μM BAM (8-22) indicated by the labelled line)

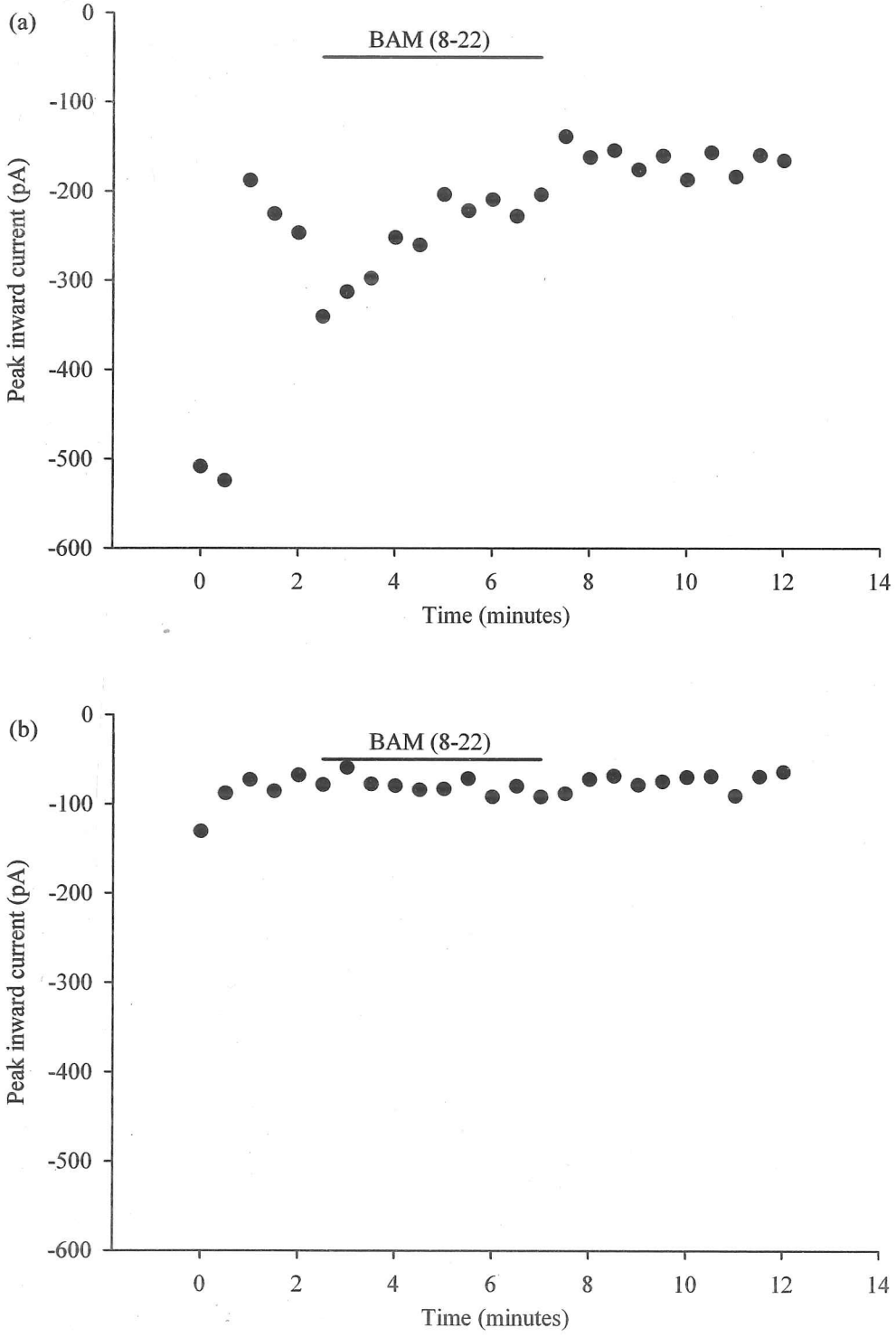

Figure 5.3 Examples of neurones exposed to BAM (8-22) that were found not to be sensitised

Responses to 100 nM capsaicin are denoted by the points on the graph and the application of BAM (8-22) is indicated by the black line.

to this calculated data. Then for each neurone the confidence limits were applied to the differences between the predicted baseline and the points during the treatment period. Using a comparison of the actual traces with this analysed data it was determined that if a neurone had a run of significant negative differences, centred away from the edges of the treatment period, then it was sensitised. This analysis showed that 2 out of 17 neurones were sensitised by BAM (8-22).

In addition to examining the peak inward current, the decay component of the response to capsaicin, as measured using a one-term, standard exponential fitted using the Simplex fitting method (with zero-shift), revealed in three cases a significant increase in value indicative of a slowed return to baseline. According to Vellani *et al.* (2001), longer applications of capsaicin result in an increased current and a longer time to return to baseline. Thus as discussed earlier, with PGE_2, a prolonged interaction of capsaicin with TRPV1 could be indicative of a sensitised receptor. Therefore, when this measurement is used, 3 out of the 17 cases are sensitised by BAM (8-22). Interestingly, only 1 of these 3 showed a significant increase in the peak inward current.

In addition to examining the peak inward current, the response of each neurone to α,β-Me-ATP was assessed in order to test for the presence of $P2X_3$ receptors. All neurones that responded to capsaicin, and were able to be tested for responsiveness to α,β-Me-ATP (n=13), did respond. Thus all TRPV1 expressing neurones in these experiments were also found to express $P2X_3$ receptors. This is different from the calcium-imaging experiments where some experiments showed TRPV1 expressing neurones to be non-responsive to α,β-Me-ATP. This, however, may be as a result of a lower n number.

The effect of BAM (8-22) in the presence of Ro-31-8220

Because the calcium imaging experiments from the previous chapter showed that PKC was involved in the sensitisation of nociceptive neurones by BAM (8-22), the effect of BAM (8-22) on TRPV1 was also examined in the presence of the PKC selective inhibitor Ro-31-8220. The same protocol was followed as for examining the effect of BAM (8-22) except that 500nM Ro-31-8220 was present throughout. Figure 5.4(a) contains an example plot of a neurone, which although moderated by the presence of Ro-31-8220 when compared with Figure 5.2, was still determined to be sensitised by BAM (8-22). Figure 5.4(b) displays individual capsaicin-evoked inward currents from the fifth and eighth capsaicin additions. Again, 2 out of 17 neurones were seen to sensitise in response to the application of BAM (8-22).

Therefore, these experiments do not support PKC involvement in the sensitisation of TRPV1 by BAM (8-22). The number of experiments is too small, however, to allow definite conclusions to be drawn.

Comparison of cell diameter with sensitisation

Figure 5.5 displays a comparison of cell diameter with sensitisation by BAM (8-22). The figure reveals that there is no cluster of neurones around a specific cell diameter, and instead those neurones which do sensitise appear spread across the range of cell diameters for neurones in these experiments. Again a greater *n* number would permit a more accurate assessment of whether particular cell diameters are sensitised.

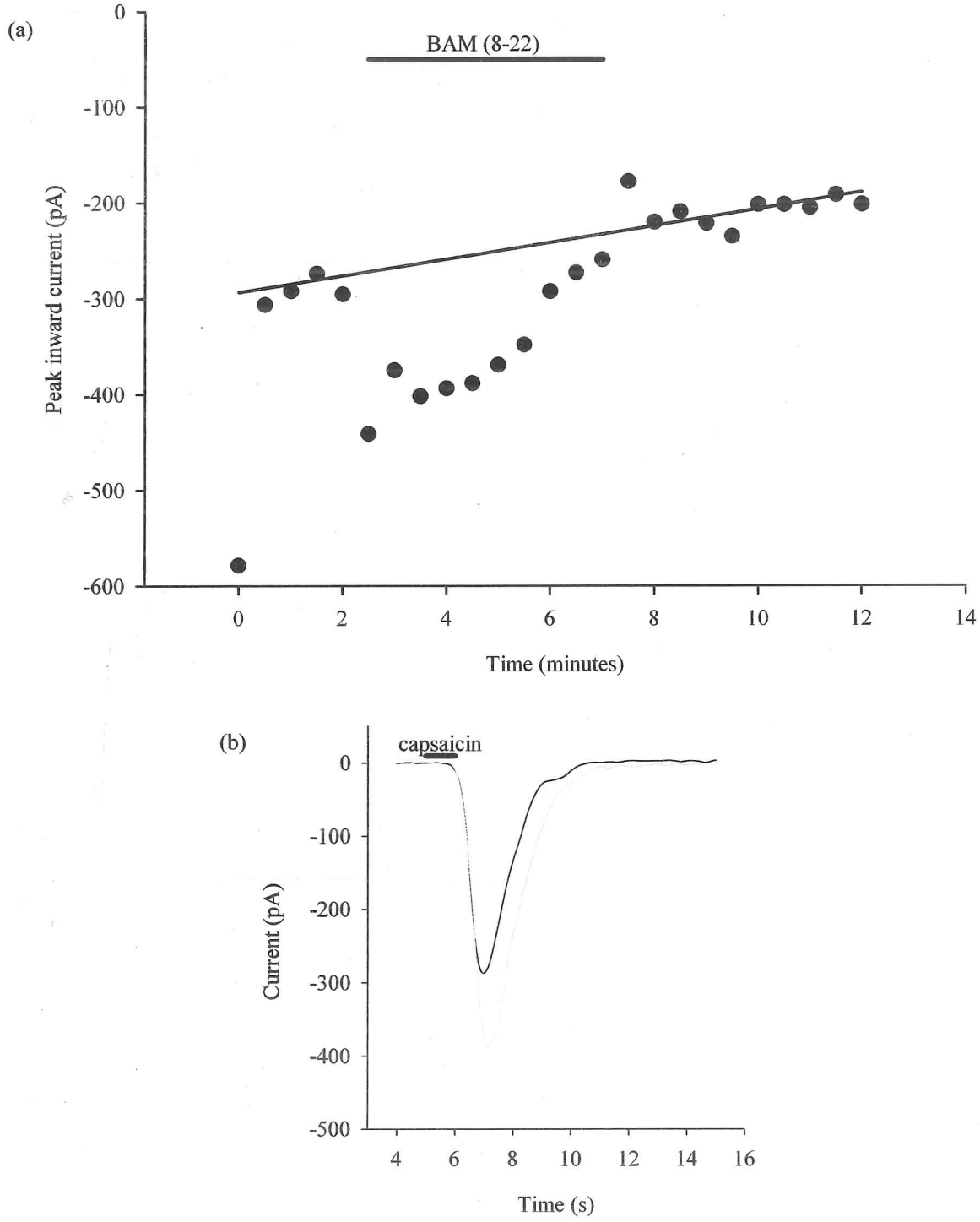

Figure 5.4 An example of a neurone where the sensitising effect of BAM (8-22) is moderated by the presence of the PKC selective inhibitor, Ro-31-8220 (500 nM).

(As for figure 5.2 except that Ro-31-8220 is present throughout)

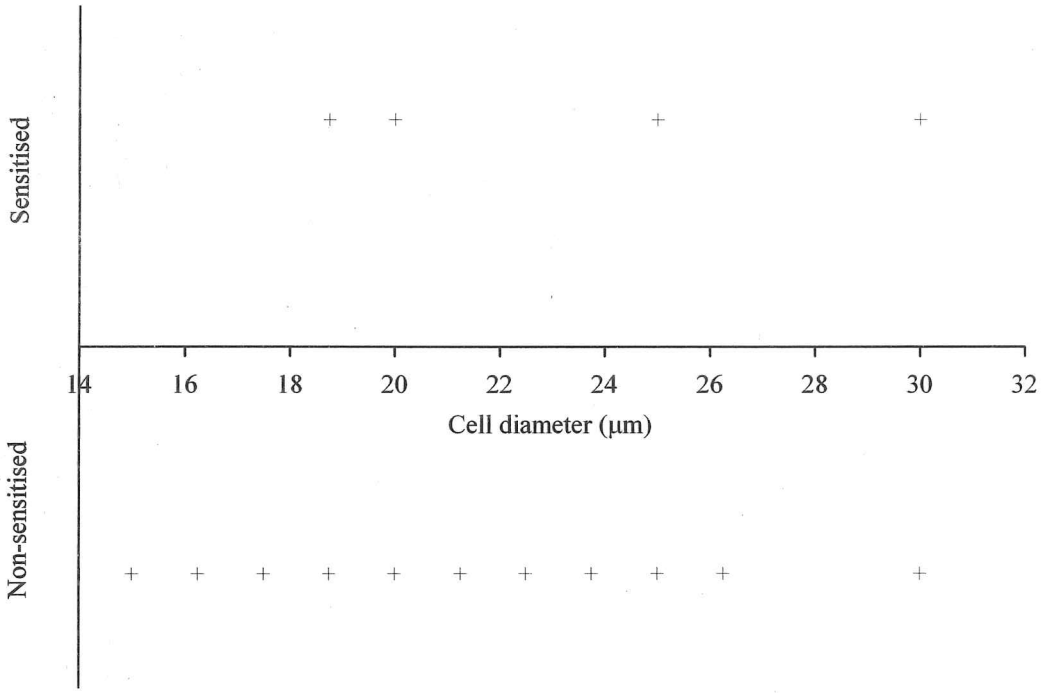

Figure 5.5 Sensitisation by BAM (8-22) occurs for neurones across the spectrum of those expressing TRPV1.

A graph of DRG neurones exposed to BAM (8-22) where the longest axis was measured to provide the cell diameter (in μm).

Conclusion

The above patch clamp experiments show that the sensitising effect of BAM (8-22) involves an enhancement of the response of TRPV1 in a subset of nociceptive neurones. The percentage of neurones sensitised in this manner (12%) is similar to that seen in the calcium imaging experiments, but unlike in those experiments no clear evidence was found for an inhibition of sensitisation in the presence of the PKC inhibitor Ro-31-8220.

There is a slight caveat associated with these data in that, in order to maximise the number of neurones from which recordings could be obtained, DRG neurones of a specific morphology were selected to be patched given their increased likelihood of expressing TRPV1 (based upon previous work by Paolo Cesare). Thus the percentage reported for the patch clamp experiments is biased by this fact.

Chapter 6

Discussion

6.1 Vanilloid receptor (TRPV1) activation by capsaicin

Capsaicin is known to cause an increase in intracellular calcium levels in a subpopulation of cultured dorsal root ganglion (DRG) neurones (Dray *et al.*, 1990; Akerman and Gronblad, 1992; Cholewinski *et al.*, 1993; Greffrath *et al.*, 2001; Savidge *et al.*, 2001) as a result of the activation of the TRPV1 receptor (Caterina *et al.*, 1997). The increase in intracellular calcium level depends on the presence of extracellular calcium (Cholewinski *et al.*, 1993; Greffrath *et al.*, 2001; Savidge *et al.*, 2001) with the major influx being through voltage-gated calcium channels, as well as an approximate 25% influx through the TRPV1 receptor itself (Akerman and Gronblad, 1992; Greffrath *et al.*, 2001). Some reports have also suggested that the release of calcium from intracellular stores plays a small but significant role (Cholewinski *et al.*, 1993; Greffrath *et al.*, 2001). The return of the intracellular calcium level to a basal level is carried out by mechanisms contributing to intracellular calcium homeostasis (Greffrath *et al.*, 2001). Based upon preliminary experiments and published concentration-response data (Cholewinski *et al.*, 1993), 100 nM capsaicin was selected to activate the TRPV1 receptors as it was close to the published half maximal effective concentration (EC_{50}) value.

In order to examine the modulation of the responsiveness of DRG neurones expressing the TRPV1 receptor, while removing as far as possible the complicating factor of tachyphylaxis of the response, neurones were repeatedly exposed to 100 nM capsaicin. An inter-stimulus interval of four minutes was selected based upon work previously carried out in the lab by Jennifer Bonnington (Bonnington and McNaughton, 2003). Tachyphylaxis on repeated capsaicin additions (as seen in Figure 3.2) has been demonstrated before in other studies (Docherty *et al.*, 1996; Koplas *et al.*, 1997) and is believed to be as a result of the activation of calcineurin, by increased intracellular calcium levels, which then dephosphorylates and

desensitises the TRPV1 receptors (Docherty *et al.*, 1996). Once a steady response had been reached a clearer interpretation of the effect of a treatment could be made.

6.2 Cannabinoid receptors

Effect of the activation of cannabinoid receptors on nociceptive neurones

In order to examine if the activation of cannabinoid receptors on nociceptive neurones affects the responsiveness of the neurones it was necessary to select a suitable cannabinoid receptor agonist. The synthetic cannabinoid WIN 55,212-2, known for efficacy at both CB_1 and CB_2 receptors (Pertwee, 2000), was selected rather than the endocannabinoid anandamide (which features in many recent cannabinoid studies) because of the potential complication that could be introduced by its activation of TRPV1 receptors (Zygmunt *et al.*, 1999; Smart *et al.*, 2000; Tognetto *et al.*, 2001). Treatment with 100 nM WIN 55,212-2 for two minutes was not found to have any significant effect on the responsiveness of non-sensitised nociceptive neurones to capsaicin, as measured by the increase of intracellular calcium concentration. A significant effect following CB_1 receptor stimulation, however, has been seen in previous studies on evoked increases in intracellular calcium levels in cultured DRG neurones (Tognetto *et al.*, 2001; Millns *et al.*, 2001) as well as on capsaicin-evoked calcitonin gene related peptide (CGRP) release (Ahluwalia *et al.*, 2003). The activation of CB_1 receptors on cultured DRG neurones was also shown by Ross *et al.* (2001) to cause an inhibition of voltage-gated calcium channels. There are several reasons the present study has not revealed a significant inhibition of nociceptor responsiveness following cannabinoid receptor activation. One of the most obvious is the nature of the analysis. When determining if a

neurone had desensitised, there is a much smaller range of values that it can be in order for it to be classified as desensitised. Thus the analysis employed is less sensitive to detecting desensitisation. Ross *et al.* (2001) saw a significant effect with 100 nM WIN 55,212-2 on voltage-gated calcium channels in DRG neurones, so it is unlikely that the concentration of WIN 55,212-2 was insufficient, although Ross *et al.* (2001) utilised a longer treatment period. Interestingly, Khasabova *et al.* (2002) did find WIN 55,212-2 to have an inhibitory effect on K^+-evoked increases in intracellular calcium concentration. In this case, however, the much higher concentration of 1 μM WIN 55,212-2 was employed. Furthermore, the neurones in which this effect was seen rarely responded to capsaicin.

Much of the behavioural work discussed in the introduction has suggested that the effects of cannabinoid receptor activation in the periphery are antihyperalgesic rather than analgesic. The effect of WIN 55,212-2 on TRPV1 in neurones in which the response to capsaicin had been potentiated by prior application of PGE_2 was therefore studied.

Effect of PGE_2 on nociceptive neurones

Exposing cultured dorsal root ganglion neurones to 1 μM PGE_2 for two minutes resulted in both a visible sensitisation of the response of a subset of neurones to the application of capsaicin, and a sensitisation of a significant proportion of nociceptive neurones when compared with control data. The percentage of neurones found to be sensitised (approximately 21% when the transient height was examined and 31% when the transient area was examined) is slightly less than the 37% previously reported by Pitchford and Levine (1991). Such a sensitisation is believed to be mediated on a molecular level by the cyclic 3',5'-adenosine monophosphate/Protein Kinase A (cAMP/PKA) modulation of the ion

channels involved in mediating the tetrodotoxin-resistant (TTX-R) sodium current—to increase their unitary current and lower the membrane potential threshold at which they activate (England *et al.*, 1996; Gold *et al.*, 1996b). An increase in the mean channel activity of TRPV1 has also been reported (Lopshire and Nicol, 1998). Both of these modulations could be expected to result in an increase in the elevation of the intracellular calcium levels evoked by capsaicin by making the neurone more responsive—in a general sense by the TTX-R sodium current modulation, and specifically by enhancing the response of the TRPV1 receptor (Caterina *et al.*, 1997).

The duration of the sensitisation was assessed in these experiments and was generally found to be limited to the capsaicin addition immediately after the treatment with PGE_2. The effect of PGE_2 was therefore rapid in onset, and also recovered from rapidly. The phenotype of the neurones sensitised by PGE_2 in these experiments was assessed by examining responsiveness to a $P2X_3$ agonist. In order to do this, the selective agonist α,β-methyleneadenosine 5'-triphosphate (α,β-Me-ATP) was employed. In the present experiments, approximately 43% of neurones responded to capsaicin, and 44% of neurones responded to α,β-Me-ATP. Previous studies have shown that both *in vitro* (Wood *et al.*, 1988) and *in situ* (Guo *et al.*, 1999) at least 50% of DRG neurones express TRPV1. Although this is very species dependent, the neurones in the present study are close to the expected phenotype. α,β-Me-ATP has been shown to selectively activate $P2X_3$ receptors in cultured neonatal rat DRG neurones (Robertson *et al.*, 1996; Rae *et al.*, 1998). The EC_{50} value for α,β-Me-ATP to evoke transient, inward currents in these neurones has been shown to be approximately 2μM (Robertson *et al.*, 1996) thus the 100μM employed in the present study should be more than sufficient to activate all the $P2X_3$ receptors present. Studies examining the percentage of neurones expressing $P2X_3$ receptors do not, however, all agree. Both Grubb & Evans (1999) and Guo *et al.* (1999) indicate that approximately 80% of adult rat DRG neurones could be

expected to express $P2X_3$ receptors. This contrasts with Vulchanova *et al.* (1997) who suggested that expression is limited to 40% of neurones in rat and Chen *et al.* (1995) where approximately 56% of rat DRG neurones were found to express levels of $P2X_3$ transcript above background signals. The present study, using neonatal neurones, found 44% of neurones which could be expected to express $P2X_3$ receptors which is more in line with the results from Vulchanova *et al.* (1997). The explanations offered by Guo *et al.* (1999) for the discrepancies between their findings and Vulchanova *et al.* (1997), such as selective survival of smaller diameter neurones, strain differences, and functional studies providing a more sensitive assay for receptor expression do not hold in the present study, because as in Guo *et al.* (1999), the present study selects smaller diameter neurones, employs Wistar rats, and involves a functional study. The most likely source of difference is the use of neonatal rats in the present study. The present study found approximately 38% of TRPV1 expressing neurones also express functional $P2X_3$ receptors, much less than the 75% reported by Guo *et al.* (1999). When sensitisation by PGE_2 was examined there did not appear to be any bias towards those neurones which did express $P2X_3$.

Approximately 10% of the neurones analysed responded to capsaicin only following treatment with PGE_2. Such a recruitment of 'silent' sensory neurones (Michaelis *et al.*, 1996) has indeed been seen after PGE_2 exposure before (Mense, 1981; Stucky *et al.*, 1996) and could aid to promote central sensitisation at the dorsal horn (which underlies secondary hyperalgesia). Such recruitment could be mediated by sensitisation of the TRPV1 receptor (Lopshire and Nicol, 1998). Also, a subsequent activation of voltage-gated calcium channels would be promoted by sensitisation of the TTX-R sodium current (England *et al.*, 1996; Gold *et al.*, 1996b).

In conclusion, sensitisation was observed following treatment by PGE_2. The transient nature of the recruitment of 'silent' neurones, and the fact that the sensitisation evoked by

PGE_2 was seen to disappear in subsequent capsaicin additions, may suggest that calcium entry activates phosphatases (e.g. calcineurin) which antagonise the cAMP/PKA mediated PGE_2 sensitisation.

Possible mechanisms of antihyperalgesia

Sensitisation of nociceptive neurones can occur via a variety of mechanisms, and thus antihyperalgesia can also occur via a range of mechanisms. Sensitisation can be mediated by ligands directly interacting with ion channels, as well as effects mediated via second messenger signalling cascades. As discussed in the introduction, protons can directly modulate the responsiveness of TRPV1. Furthermore, 5-hydroxytryptamine (5HT) (Cardenas *et al.*, 2001), tumour necrosis factor α (TNFα) (Junger and Sorkin, 2000), nerve growth factor (NGF) (Shu and Mendell, 1999), histamine (Mizumura *et al.*, 1994), prostaglandins (England *et al.*, 1996) and bradykinin (Burgess *et al.*, 1989) all modulate the sensitivity of nociceptors through intracellular signalling mechanisms. The two major signalling pathways involved in sensitisation are the protein kinase C (PKC) and PKA pathways. Therefore in order to have an antihyperalgesic effect one of three things must occur:-

(a) the agonist is sequestered from its site of action,

(b) there is a direct attenuation of the transducer on which the sensitising effect is occurring (e.g. as when calcineurin causes the desensitisation of TRPV1), or

(c) there is an attenuation of the sensitising pathway itself.

Effect of cannabinoid receptor activation on sensitised nociceptive neurones

Treatment with the cannabinoid receptor agonist WIN 55,212-2 at the same time as PGE_2 was not seen to significantly attenuate the sensitisation previously seen with PGE_2. This may be as a result of either too low a concentration of cannabinoid agonist being employed or too short an incubation. Sensitisation by PGE_2, however, is only one facet of the sensitisation that can occur with nociceptive neurones. Reports from the Rice group (Farquhar-Smith *et al.*, 2002; Farquhar-Smith and Rice, 2003) show that the endogenous cannabinoid system can modulate the nerve growth factor (NGF)-mediated components of inflammatory hyperalgesia. Furthermore, the *in vivo* effects may be as a result of the modulation of non-neuronal cells such as an inhibition of the release of inflammatory mediators from mast cells. Alternatively, the effects of cannabinoid receptor activation may centre around the inhibition of voltage-gated calcium channels as seen by Ross *et al.* (2001) and Khasabova *et al.* (2002). The different nature of the assay used in the present study from others employed in published reports could explain why no significant antinociceptive effect was seen. This suggestion is perhaps supported by the mild attenuation seen in the model of sensitisation using PGE_2 employed here, which may provide a more sensitive assay. The antihyperalgesic effect could thus be due to an inhibition of neurogenic inflammation which would not be seen in this experimental protocol.

The neuronal phenotype in the present experiments was assessed and approximately 42% of neurones were found to respond to 100 nM capsaicin, and 36% were found to express $P2X_3$ receptors. A similar result was obtained when WIN 55,212-2 was absent, though the number of neurones responding to α,β-Me-ATP was slightly reduced. This time, however, the distribution of those neurones which were sensitised by PGE_2 was biased towards those neurones which did not express $P2X_3$ receptors. Thus it appears that the neurones that are no

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Implications of results with respect to localisation studies

The lack of a statistically significant effect with the cannabinoid receptor agonist in the results presented here could be due to the localisation of the CB_1 receptors. There have been several reports examining the localisation of cannabinoid receptors at the peripheral and spinal level, and these reports have not been consistent. Furthermore, the results obtained with respect to DRG neurones *in vitro* compared with *in situ* differ. In general, the results obtained *in vitro* (Ahluwalia *et al.*, 2000; Ahluwalia *et al.*, 2002) suggest that the majority of CB_1-like immunoreactivity identified in DRG neurones was associated with markers for nociceptive neurones. In particular, almost all neurones displaying TRPV1-like immunoreactivity were found to display CB_1-like immunoreactivity (Ahluwalia *et al.*, 2000). Such a distribution would suggest that a modulation of TRPV1-expressing DRG neurones by a cannabinoid agonist could be expected *in vitro*. The lack of an effect could be due to two reasons: either functionally there is no effect, or, in fact, these neurones do not in fact express cannabinoid receptors. The former possibility is perhaps suggested by the functional study carried out by Khasabova *et al.* (2002) where it was found that despite the presence of CB_1-like immunoreactivity, a cannabinoid agonist generally had no effect on the responsiveness of these neurones. This is in contrast to the finding by Millns *et al.* (2001) who reported that a cannabinoid agonist could attenuate the capsaicin-induced calcium response in DRG neurones. The latter possibility is suggested by the numerous *in situ* studies that have shown a low level of co-localisation between CB_1 receptors and markers for nociceptive neurones (Hohmann and Herkenham, 1998). The most recent of these by Bridges *et al.* (2003) reported that only about 7% of TRPV1 expressing cells were found to express CB_1 mRNA. The majority of DRG neurones that express CB_1 receptors seem to be myelinated, non-TRPV1 expressing A-

(continued)

longer sensitised when WIN 55,212-2 is present are more likely to express $P2X_3$ receptors. Such a result ties in with the localisation study by Ahluwalia *et al.* (2002) in which they found that amongst nociceptive neurones, CB_1-receptor-like immunoreactivity was more likely to be present in the isolectin B4 (IB_4) binding population which express $P2X_3$ receptors (Julius and Basbaum, 2001).

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6.3 Sensory Neurone Specific Receptors (SNSRs)

Effect of the activation of SNSRs on nociceptive neurones

Sensory neurone specific receptors (SNSRs) are a novel group of GPCRs that were reported by Lembo *et al.* (2002) to be found in a subset of small diameter sensory neurones. Given reports by Cao *et al.* (2003) and Grazzini *et al.* (2004) that SNSR activation resulted in enhanced pain behaviour, and given that Lembo *et al.* (2002) and Zylka *et al.* (2003) found colocalisation of SNSRs with TRPV1, the effect of the SNSR selective agonist BAM (8-22) on the response of nociceptive neurones expressing TRPV1 was examined. Grazzini *et al.* (2004) found that BAM (8-22) had an EC_{50} of 120±15 nM at rat SNSR1, and so 5 μM BAM (8-22) was selected in order to ensure a supramaximal dose was present. A four minute application of this treatment resulted in a significant number of neurones (approximately 15%) sensitising compared with control data. Thus a rather low percentage of TRPV1 expressing neurones appear to express functional SNSRs. Zylka *et al.* (2003) showed that 50.9±8.0% of TRPV1 expressing neurones also expressed rMrgD (see Chapter 1 for a comment on the nomenclature) and that 1.1±1.1% expressed rMrgC. Thus there appears not to be a direct correlation between rMrg expression and sensitisation. There could be several

fibre neurones (Bridges *et al.*, 2003). Such a result is supported by the results of Hohmann and Herkenham (1998), and Hohmann and Herkenham (1999b) who noted that the majority of CB_1 mRNA expressing cells were those with fibres of larger diameter. Indeed Khasabova *et al.* (2002) noted that the activation of CB_1 receptors inhibited the response of intermediate diameter neurones that rarely responded to capsaicin.

Ahluwalia *et al.* (2002) discussed reasons as to why different immunohistochemical studies gave different answers. One of the reasons for a difference may be the presence of more than one subtype of cannabinoid receptor in DRG neurones.

In addition to the disparity in the phenotype of DRG neurones that express CB_1 receptors, the expression of CB_1 receptors by DRG neurones is disputed by some reports. Hohmann and Herkenham (1999a) identified CB_1 receptors in DRG neurones using radioligands. Hohmann *et al.* (1999) and Salio *et al.* (2002) reported the presence of cannabinoid receptors both pre- and post-synaptically in the spinal dorsal horn. Sañudo-Peña *et al.* (1999) also noted in tissue sections that CB1-like immunoreactivity was present in DRG as well as spinal cord. Farquhar-Smith *et al.* (2000), however, found that whereas there was co-laminal localisation in the dorsal horn with nociceptive primary afferents, there was little co-localisation at the fibre level. The majority of CB_1-like immunoreactivity was found on spinal interneurones suggesting that cannabinoids have a modulatory role at the level of the dorsal horn rather than at the level of primary sensory afferents. Salio *et al.* (2002) note that the difference may be due to a heterogeneity of cannabinoid binding sites in the dorsal horn. The result by Farquhar-Smith *et al.* (2000) is in contrast to the functional studies that show that cannabinoid agonists have a modulatory role in the periphery (Richardson *et al.*, 1998a; Ko and Woods, 1999; Johanek *et al.*, 2001).

(continued)

reasons for this. Zylka *et al.* (2003) examined Sprague-Dawley adult dorsal root ganglia (DRG) whereas this present study utilises Wistar neonatal rats. Not only is there a possibility of strain variation, but also the chance of changed expression with age. The phenotype of nociceptive neurones is known to change over the course of the first two weeks of the life of a rat (Roy and Narahashi, 1992). There is also the difficulty in knowing to what extent BAM (8-22) activates rMrgD which is by far the most abundant receptor in TRPV1 expressing neurones. Grazzini *et al.* (2004) examined the effect of BAM (8-22) on rMrgC and found it to be a full agonist. BAM (8-22) has not been tested on rMrgD, but Grazzini *et al.* (2004) noted that it was inactive at rMrgA (which has the closest match to rMrgC at the amino acid level) at concentrations up to 10μM. Thus an action at rMrgD could be unlikely but more work in this area is obviously needed. Furthermore, Grazzini *et al.* (2004) found through the use of radioligand binding that only a single population of binding sites existed in the spinal cord and the periphery suggesting the lack of another BAM (8-22) binding site. If this is so then it seems anomalous that 15% of neurones sensitise rather than the approximately 1% seen to express rMrgC. It seems that the most likely explanation is that the phenotype of neonatal neurones with respect to these receptors is different from adult DRG, with perhaps a greater percentage expressing rMrgC.

The fact that a percentage of neurones is seen to sensitise following treatment with BAM (8-22) fits with the *in vivo* electrophysiology (Cao *et al.*, 2003) and behavioural data (Grazzini *et al.*, 2004) showing that the activation of rat SNSRs results in hyperalgesia. The present results showing that this occurs at the level of the neurone also links in with the finding by Grazzini *et al.* (2004) that the heat hyperalgesia seen in behaviour studies in response to the activation of rSNSR1 is not secondary to inflammatory effects.

As well as examining the effect of BAM (8-22) treatment on the height of the capsaicin-evoked calcium transient the effect on the area of the evoked calcium transient was also

The lack of an effect of cannabinoid receptor activation in the model of sensitisation could also be as a result of the distribution of cannabinoid receptors as Hohmann *et al.* (1999) note the difference in cannabinoid receptor and mu opioid receptor distribution with respect to capsaicin sensitivity. Hohmann and Herkenham (1999b) noted that the difference in distribution of CB_1 and mu opioid receptors could underlie different roles. Cannabinoid receptors were found on fibres with a range of diameters, whereas mu opioid receptors were only expressed on thin diameter fibres. Such a difference in distribution could explain why the activation of mu opioid receptors has been seen to modulate the sensitisation mediated by PGE_2 (Gold and Levine, 1996) whereas cannabinoid receptors were not found here to modulate this sensitisation.

investigated as this could be expected to provide some indication of a direct involvement of TRPV1. There was, however, no significant difference in this parameter suggesting that the neuronal components affected most by the sensitisation were those involved in depolarisation (i.e. voltage-gated ion channels).

Under inflammatory conditions some normally unresponsive primary afferent neurones become responsive to chemical stimuli (Michaelis *et al.*, 1996; Stucky *et al.*, 1996). This recruitment of 'silent' primary afferent neurones could be expected to contribute to inflammatory hyperalgesia. Thus the finding in the present study that treatment with BAM (8-22) results in the recruitment of 5.10% of 'silent' nociceptors provides another mechanism by which BAM (8-22) can result in the hyperalgesia reported by Cao *et al.* (2003) and Grazzini *et al.* (2004).

No response to the application of BAM (8-22) itself was seen, ruling out a direct effect of BAM (8-22) on intracellular calcium levels. This result differs from previous reports in which SNSR was heterologously expressed. SNSR agonists were shown to cause a direct calcium release from intracellular stores in these expression systems (Lembo *et al.*, 2002; Han *et al.*, 2002; Grazzini *et al.*, 2004). The release was not affected by pertussis toxin pre-treatment (Lembo *et al.*, 2002; Grazzini *et al.*, 2004) but was completely inhibited by a PLC inhibitor (Han *et al.*, 2002) thus suggesting that rSNSR1 is a $G_{\alpha q}$ coupled-receptor (Grazzini *et al.*, 2004). Han *et al.* (2002) further found that SNSR activation triggered calcium release from intracellular stores via an IP_3-dependent mechanism resulting in a store activated influx of extracellular calcium. The lack of a similar finding in DRG neurones may be the result of a lack of sufficient receptor density to activate PLC to produce enough IP_3 to release calcium.

Given the fact that the present study shows that the sensitisation is limited to the capsaicin-evoked calcium transient immediately after the treatment with BAM (8-22), and that

sensitisation of such a response is often limited by phosphatases (Docherty *et al.*, 1996), it seemed prudent to investigate the involvement of protein kinases in this sensitisation.

Involvement of protein kinases in the sensitisation mediated by BAM (8-22)

Initially the general kinase inhibitor staurosporine was employed to test for possible protein kinase involvement. A concentration of 200 nM was selected based upon previously published work (Chen and Penington, 1996). Staurosporine was found to suppress the sensitisation by BAM (8-22) such that it was no longer significantly different from control. Thus there was evidence that protein kinases were involved in mediating the sensitisation by BAM (8-22).

The effect of the PKC selective inhibitor Ro-31-8220 was next investigated. Selective inhibition of PKC was found to attenuate significantly the sensitisation mediated by BAM (8-22) thus indicating an involvement of PKC in the sensitising pathway.

There is some evidence that the PKA-selective inhibitor H-89 may slightly attenuate the sensitisation by BAM (8-22) but the effect does not reach significance. Therefore there is unlikely to be significant involvement of the cAMP/PKA pathway in mediating the effect of BAM (8-22).

Given that PKC was found to play a significant role in the mechanism of sensitisation caused by BAM (8-22), the isoform of PKC which may be involved was investigated. Cesare *et al.* (1999a) investigated the role of the various PKC isoforms found in DRG in the sensitising pathway linking the B_2 receptor to TRPV1. Five isoforms were found in cultured neonatal rat DRG neurones: PKC- β_I, β_{II}, δ, ε, and ζ. Olah *et al.* (2002) found that a culture of embryonic rat DRG neurones contained the isoform PKC-α too, and that it was involved in

the sensitisation of TRPV1. In the present study only the δ, ε, and ζ isoforms were examined. None of these isoforms were seen to be translocated by treatment with BAM (8-22). Therefore if PKC is involved in the sensitisation, as suggested by the effects of Ro-31-8220, it must either be one of the untested isoforms, or because a signalling pathway was activated without translocation (one such pathway is discussed in the overall conclusions below).

Modulation of TRPV1 by BAM (8-22)

As discussed earlier, and noted by both Greffrath *et al.* (2001), and Bonnington and McNaughton (2003), when cultured DRG neurones are stimulated with capsaicin, the resultant increase in intracellular calcium may be from three different sources: flow through TRPV1; release from intracellular stores; and influx through voltage gated calcium channels. Any sensitisation seen in a calcium imaging assay would therefore need to be investigated further in order to establish the molecular components that were sensitised. Bonnington and McNaughton (2003) utilised non-TRPV1 neuronal depolarising agents (adenosine triphosphate (ATP) and KCl), as well as lidocaine to inhibit voltage-gated sodium channels, and thapsigargin to empty intracellular calcium stores, and they established that the sensitising action of NGF was directly on TRPV1 and not via other indirect means. This technique therefore proved to be a simple and direct means of monitoring sensitisation of TRPV1 by NGF, and allowed a large neuronal population to be assessed.

Given that inflammatory mediators have been shown in the past to sensitise nociceptive neurones via alteration of the characteristics of TRPV1 (Lopshire and Nicol, 1997), the direct effect of BAM (8-22) on the response of TRPV1 to capsaicin was investigated by means of the patch clamp technique. This technique allows individual neurones to be voltage clamped

and the currents flowing across their membranes to be monitored. Use of the agonist, capsaicin, allowed the selective activation of only TRPV1 ion channels, and thus the currents measured will be due to flow through TRPV1 ion channels. The positive aspect of the patch clamp method is that it avoids the need for the substantial additional calcium imaging experiments reported in Bonnington and McNaughton (2003). The negative, discussed later, is the lower *n* number that can be generated within a reasonable time frame.

Based upon the criteria for peak current (discussed in Chapter 5) used to record if a neurone had been sensitised, 2 out of 17 of the neurones examined were found to be sensitised (compared with none in control experiments). This represents approximately 12% of neurones being sensitised which is not dissimilar from the percentage found by the calcium imaging technique. Obviously, because of limitations with data collection, the *n* number is lower than desirable for an accurate reading of the percentage of neurones that sensitise.

The effect of selectively inhibiting PKC was also investigated using the patch clamp technique, but was not seen to have any effect. Further experiments or the use of different techniques are necessary to confirm this, as the *n* number is rather small.

Phenotype of neurones sensitising to BAM (8-22)

The neuronal phenotype of the neurones present in the culture of dorsal root ganglion neurones was also investigated. Overall in these cultures approximately 49% of neurones were seen to respond to 100 nM capsaicin which is as expected based on a previous study (Wood *et al.*, 1988). Approximately 56% of neurones were found to respond to the $P2X_3$ selective agonist α,β-Me-ATP. This correlates well with the approximately 56% of DRG neurones that Chen *et al.* (1995) found to express levels of $P2X_3$ transcript above background

signals. Those neurones being responsive to α,β-Me-ATP, and thus likely to express $P2X_3$, predominated in the experiments in which a sensitising effect of BAM (8-22) was seen. Such a finding correlates with the finding by Zylka *et al.* (2003) that in the rat all Mrg positive neurones express the purinergic receptor $P2X_3$. The lack of complete correlation may be due to animal age, differences between *in vivo* and in *vitro* conditions, and rat strain.

The nature of the neurones sensitised was also assessed in the patch clamp experiments by comparing cell diameter with whether or not the neurone was sensitised or not. Ueno *et al.* (1999), in a study of responses to ATP, classified their cells as: small cells, less than 25 μm in diameter; medium cells, between 25 and 35 μm; and large cells, greater than 35 μm. The neurones seen to be sensitised in the present study were split between small and medium sized cells, both of which could be expected to respond to α,β-Me-ATP (Ueno *et al.*, 1999). This is in line with the expression pattern of nociceptive markers expected to be shown by neurones expressing SNSRs.

Summary

The activation of SNSRs on cultured nociceptive neurones by the selective agonist BAM (8-22) has been shown to sensitise a subset of these neurones. This sensitisation is predominantly in nociceptive neurones expressing the $P2X_3$ receptor. The molecular mechanisms underlying the sensitisation have only partially been elucidated, but BAM (8-22) has been shown here to sensitise neurones via a pathway involving PKC.

6.4 Overall Conclusions

The present study furthers our knowledge of the intracellular pathways involved in the sensitisation of nociceptive neurones. In the cannabinoid study, the lack of effect of cannabinoid receptor activation on the sensitisation of the nociceptive neurones by PGE_2 suggests that the antihyperalgesic effects of cannabinoid receptor activation that are observed *in vivo* are due to an alternative intracellular pathway than the hypothesised modulation of cAMP/PKA. Furthermore, the lack of an effect in the PGE_2 model of sensitisation suggests that PKC-ε translocation (personal communication, V. Vellani) is not involved either. Reports from the Rice group (Farquhar-Smith *et al.*, 2002; Farquhar-Smith and Rice, 2003) show that the endogenous cannabinoid system can modulate the NGF-mediated components of inflammatory hyperalgesia. Moreover, CB_2 receptor activation by palmitoylethanolamide (PEA) was seen to reduce NGF-mediated neutrophil influx which could be linked to thermal hyperalgesia. In comparison, CB_1 receptor activation by anandamide resulted in a direct neuronal antihyperalgesic action in the case of the NGF induced thermal hyperalgesia. Farquhar-Smith and Rice (2003) conclude that this suggests a therapeutic site of analgesic action separable from central side effects.

When the SNSR study is examined, PKC is seen to be involved in the sensitisation by BAM (8-22) of those neurones expressing TRPV1. No translocation of the PKC isoforms examined (δ, ε and ζ) was seen in response to BAM (8-22) application. Two possibilities exist for this (personal communication X. Zhang): the isoforms β_I and β_{II} may be involved, and as they are already translocated they cannot be examined in this assay, or the PKC δ isoform may be involved in a signalling pathway where no translocation is necessary. Such a pathway is discussed below. Further investigation into the intracellular signalling pathways is necessary

in order to provide a clearer idea. For example, at which signalling point does cannabinoid receptor activation block the NGF sensitisation?

The 'silent' nociceptors mentioned in the course of this study need further work to establish if they are really silent. For example, the responses may have been just below the threshold for measurement of the calcium transient height. A greater capsaicin concentration would ensure all those neurones which could respond prior to sensitisation, did.

One manner in which the signalling pathways could be investigated would be to transfect plasmids of the cannabinoid receptors into a heterologous cell line lacking PKC-δ (such as those used by Brandt *et al.* (2003)). This would allow the involvement of PKC-δ to be investigated even if it does not translocate in response to activation. In fact, Brandt *et al.* (2003) found that PKC-δ via protein tyrosine phosphatase (PTP) alpha resulted in the activation of the non-receptor tyrosine kinase, Src. A more recent study by Jin *et al.* (2004) revealed that TRPV1 is a potential target for cellular tyrosine kinase-dependent phosphorylation and this includes Src. Further work to examine if NGF sensitisation is mediated via Src is necessary. Another manner in which the involvement of non-translocating PKC isoforms could be examined, would be to attenuate isoform expression in DRG neurones e.g. by agonist-induced downregulation as in Olah *et al.* (2002). This could be used both with respect to cannabinoid receptors and SNSRs.

Further experiments would include utilising a phospholipase C (PLC) inhibitor to confirm if the native rSNSR1 is a $G_{\alpha q}$-coupled receptor giving rise to diacylglycerol on activation. This would also allow the examination of whether a decrease in phosphatidyl inositol 4,5-bisphosphate (PIP_2) through PLC-mediated hydrolysis played a role in the sensitising effect of BAM (8-22). This could be the case given that Chuang *et al.* (2001) found that a reduction of PIP_2 levels through PLC-mediated hydrolysis could mimic the potentiating effects of bradykinin and NGF at the cellular level with respect to TRPV1. Given that nociceptive

neurones can also be sensitised via a modulation of the TTX-R sodium current, and this can be via the PKC signalling pathway (Gold *et al.*, 1998), it would be worthwhile examining if BAM (8-22) results in the sensitisation of the TTX-R sodium current. The level of expression of rat SNSR in Wistar neonatal neurones could be examined to investigate if there is a correlation with the percentage of neurones being sensitised. Finally, a fuller pharmacological investigation of the activation of the SNSRs to provide an EC_{50} for BAM (8-22) at the *in situ* receptor, as well as examining other SNSR agonists such as γ2-MSH (Grazzini *et al.*, 2004), would provide useful information. Of course, in a pharmaceutical context, the development of a selective antagonist would have potential therapeutic use.

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